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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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APR 26 2001

Docket No. SYN1

TECH CENTER 1600/2900

Group No. 1616

Examiner: Badio

Date: 4/23/01

SUBMISSION OF BRIEF AND FEE FOR APPELLANT

Respectfully submitted,

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Reg. No. 35,507

Appellants' Attorney

Dated: April 23, 2001



APR 26 2001

TECH CENTER 1600/2900

## Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF  
Moriarty, R.M.; Penmasta, R.;  
Guo, L.; Rao, M.S.;  
and Mehta, R.G.

SERIAL NO.: 09/008,957

FILED: Jan. 20, 1998

TITLE: 1 $\alpha$ -HYDROXYVITAMIN D<sub>5</sub>,  
ITS SYNTHESIS AND USE  
IN CANCER PREVENTION  
AND THERAPY

Docket No. SYN1

Group No. 1616

Examiner: Badio

Date: 4/23/01

Hon. Commissioner of Patents and Trademarks  
Washington, D.C. 20231

## APPLICANTS' BRIEF ON APPEAL

Sir:

This is an appeal from the decision of the Primary Examiner dated November 30, 2000, as supplemented by an Advisory Action dated February 9, 2001. A Notice of Appeal accompanied by the required fee was timely filed on February 22, 2001. The statutory filing fee of \$155.00 is submitted herewith.

I. REAL PARTY IN INTEREST

OncQuest, Inc., an Illinois corporation having a principal place of business at 2201 West Campbell Park Drive, Chicago, Illinois 60612, and the assignee of the entire interest in the worldwide rights to the invention that is the subject of the present application, is the Appellant and real party in interest.

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**II. RELATED APPEALS AND INTERFERENCES**

None.

**III. STATUS OF CLAIMS**

Claims 1-19 are pending in the case. Claims 7-9 have been withdrawn from consideration as directed to a separate invention. Claims 1-6 and 10-19 stand rejected under 35 U.S.C. § 103. Appellants appeal the rejection of Claim 1 only. A copy of claim 1 is attached hereto as APPENDIX I.

**IV. STATUS OF AMENDMENTS**

This application, U.S. Serial No. 09/008,957, was filed Jan. 20, 1998, claiming a priority date of Feb. 25, 1997, the filing date of Provisional Application Serial No. 60/039,106. A Continued Prosecution Application was filed Feb. 10, 2000.

Applicants have amended various claims in their responses of July 8, 1999 and September 7, 2000. Claim 1 as listed in APPENDIX I is in the state prior to the final rejection as defined by Appellants' amendment of September 10, 2000.

**V. SUMMARY OF THE INVENTION**

Appellants' invention is the novel compound 1 $\alpha$ -hydroxyvitamin D<sub>5</sub> and analogues thereof, although the only claim at issue on appeal is directed to 1 $\alpha$ -hydroxyvitamin D<sub>5</sub>. 1 $\alpha$ -hydroxyvitamin D<sub>5</sub> has shown great promise as a chemopreventive

agent, and has significantly less calcemic activity than  $1\alpha$ -hydroxyvitamin D<sub>3</sub> or  $1\alpha$ -hydroxyvitamin D<sub>4</sub>.

#### **VI. ISSUE PRESENTED FOR REVIEW**

The issue to be decided is:

A. Whether the Examiner erred in finding Claim 1 obvious under 35 U.S.C. § 103 over Holick et al. 4,728,643, Holick et al. 5,254,538, Bishop et al. 5,763,429, or Gulbrandsen et al. 5,700,790?

Copies of the cited references are found in APPENDIX II.

#### **VII. GROUPING OF THE CLAIMS**

Group A: Claim 1 only, based upon Examiner's assertion that it is unpatentable over Holick et al. '643, Holick et al. '538, Bishop et al. '429 or Gulbrandsen et al. '790.

#### **VIII. ARGUMENT**

The following is a very brief summary of the prosecution history to date: Claim 1 is directed to  $1\alpha(\text{OH})\text{D}_5$ . The examiner rejected claim 1 as being obvious because each of the four cited references (Holick et al. '538, Holick et al. '643, Bishop et al. '429 and Gulbrandsen et al. '790) teaches a generic group of vitamin D compounds of which  $1\alpha(\text{OH})\text{D}_5$  is a member. According to the examiner, "an ordinary artisan would have the reasonable expectation that any of the species of the genus would have similar properties and, thus, the same use as the genus as a whole." (Feb. 8, 1999 Office Action) Appellants responded that

1 $\alpha$ (OH)D<sub>5</sub> has an unexpected combination of properties (antiproliferative activity coupled with lower calcemic activity) not possessed by all generic vitamin D derivatives and, therefore, claim 1 is not obvious. (July 8, 1999 Amendment)

Appellants subsequently submitted a "side-by-side" comparison of the calcemic activity for 1 $\alpha$ (OH)D<sub>5</sub> and its closest analogues, 1 $\alpha$ (OH)D<sub>4</sub> and 1 $\alpha$ (OH)D<sub>3</sub>. (Declaration of Dr. Robert Moriarty) The examiner maintained her obviousness rejections because, she stated, "the prior art indicated that the [claimed] compounds have a lower tendency or inability to cause hypercalcemia and/or hypercalcuria". (Sept. 10, 1999 Final Office Action). The only prior art relied upon by the examiner to support this argument is Bishop et al. U.S. Patent No. 5,763,429. As will now be discussed, appellants argue that claim 1 is not obvious because (1) the Bishop statement relied upon by the examiner is factually unsupportable and would not have been believed by one skilled in the art, and (2) the prior art fails to disclose or render obvious any method for synthesizing 1 $\alpha$ (OH)D<sub>5</sub>.

A. THE EXAMINER ERRED IN RELYING ON A SINGLE, SWEEPING, UNSUPPORTED STATEMENT IN ONE REFERENCE AS EVIDENCE THAT THE FAVORABLE PROPERTIES OF APPELLANTS' CLAIMED COMPOUND WOULD HAVE BEEN EXPECTED BY A PERSON OF ORDINARY SKILL IN THE ART AT THE TIME OF THE INVENTION

To establish a prima facie case of obviousness in a genus-species chemical composition situation, the examiner must find some motivation or suggestion to make the claimed compound in light of the prior art. In order to find such motivation or suggestion there must be a reasonable likelihood that the claimed compound will have the properties disclosed by the prior art teachings. (Guidelines for the Evaluation of Claims Directed to Species of Chemical Compositions Based Upon a Single Prior Art Reference)

It is not contested that the cited references teach a generic group of vitamin D derivatives that includes appellants' claimed  $1\alpha(\text{OH})\text{D}_5$  compound. However, appellants have rebutted the examiner's prima facie case by submitting evidence that shows that  $1\alpha(\text{OH})\text{D}_5$  possesses key properties (antiproliferative activity and significantly lower calcemic activity compared to the closest prior art compounds) that would have been unexpected to a person of ordinary skill in the art at the time of the invention in view of the prior art.

The examiner's position is that this lower calcemic activity is not unexpected in view of the teaching found in Bishop U.S. Patent 5,763,429 (APPENDIX II). Applicants have provided evidence that this teaching is not only incorrect, but contrary to data known at the time of the invention. Thus, the examiner

erred by interpreting a sweeping and scientifically unfounded statement in one reference (Bishop) and using it to render obvious appellants' claim 1, despite appellants' rebuttal evidence indicating the Bishop statement was wrong and would not have created a reasonable expectation in one skilled in the art that  $1\alpha(\text{OH})\text{D}_5$  had the favorable properties it has.

The Examiner's position can perhaps best be summarized by quoting from the Final Office Action:

"The cited prior art [Bishop] teach that the compounds, including the claimed compounds, act as antiproliferative agents and cell differentiation agents without significantly altering calcium metabolism. Therefore, the ordinary artisan would expect the prior art compounds not to significantly alter calcium metabolism. The ordinary artisan would also expect there to be differences in degree to which each compound encompassed by the prior art genus alters calcium metabolism. Therefore, it is the examiner's position that the results provided in the Moriarty declaration are not unexpected or unobvious..." (underlining in original)

The sole evidence offered by the Examiner that the prior art teaches that "the ordinary artisan would expect the prior art compounds not to significantly alter calcium metabolism" is found in Bishop U.S. Patent No. 5,763,429. There at col. 5, line 60 to col. 6, line 13, Bishop states that "[t]he  $1\alpha$ -hydroxyvitamin D

compounds of formula I of the present invention are those that ... have a lower tendency or inability to cause hypercalcemia and/or hypercalcuria [than the vitamin D3 compounds]."

This passage in Bishop is scientifically unsupportable and, in fact, incorrect. The quoted passage may suggest that all compounds of Bishop formula I have a lower tendency than  $1\alpha(\text{OH})\text{D}_3$  to cause hypercalcemia, but the evidence of record shows that they do not. In fact, the closest prior art compound,  $1\alpha(\text{OH})\text{D}_4$ , does not have a lower tendency than  $1\alpha(\text{OH})\text{D}_3$  to cause hypercalcemia. According to the August 24, 1994 "Declaration Under 37 CFR 1.132" of Dr. Joyce Knutson contained in the file wrapper for Knutson et al. U.S. Patent No. 5,488,120, issued Jan. 30, 1996, eleven months before Bishop's filing date of Dec. 30, 1996,  $1\alpha(\text{OH})\text{D}_4$  is "essentially equivalent to  $1\alpha$ -hydroxy Vitamin  $\text{D}_3$  ... in its ability to stimulate an increase in serum calcium" (see APPENDIX III attached hereto, paragraph 6).

A person of ordinary skill in the art at the time of Appellant's invention would have been naïve to believe Bishop's statement, or at least the examiner's interpretation of Bishop's statement, that every compound within the genus disclosed by Bishop formula I, regardless of what is substituted for R1, R2, R3, X1 and X2, would have effective antiproliferative properties and a lower tendency to cause hypercalcemia than  $1\alpha(\text{OH})\text{D}_3$ . It is unlikely that Bishop believed this himself. Knutson, named as a co-inventor on the Bishop et al. '429 patent, had data showing that  $1\alpha(\text{OH})\text{D}_4$  and  $1\alpha(\text{OH})\text{D}_3$  have essentially the same effect on

serum calcium.

Appellants submitted additional data (see "Declaration Under 37 §1.132" of Dr. Robert Moriarty in APPENDIX IV) that also shows that Bishop's statement at col. 5, lines 60-67, or at least the examiner's interpretation of Bishop's statement, is wrong. Referring to the last two lines of data in Table I of the Moriarty declaration, the data shows that  $1\alpha(\text{OH})\text{D}_4$  has a relatively high calcemic activity compared to the claimed compound  $1\alpha(\text{OH})\text{D}_5$ . Since  $1\alpha(\text{OH})\text{D}_4$  is also a species of the generic compound of Bishop formula I, this data also refutes the examiner's argument that all compounds of Bishop formula I would be expected to be useful as antiproliferative and cell differentiation agents without significantly altering calcium metabolism.

The data in the Moriarty declaration (backed up by the statistical analysis of Dr. Samad Hedayat) shows that, between  $1\alpha(\text{OH})\text{D}_5$  and  $1\alpha(\text{OH})\text{D}_4$ , two synthetic compounds,  $1\alpha(\text{OH})\text{D}_5$  is significantly less calcemic than  $1\alpha(\text{OH})\text{D}_4$ . This is an important improvement in properties, because, unlike the other known vitamin D analogues, the desirable antiproliferative activity of  $1\alpha(\text{OH})\text{D}_5$  is not offset by undesirably high calcemic activity. No one, including Bishop, anticipated that  $1\alpha(\text{OH})\text{D}_5$  would have such a favorable combination of properties.

A person of ordinary skill in the art at the time of appellants' invention would have expected differences in calcemic activity between compounds having different structures. However,

the relatively low degree of calcemic activity in  $1\alpha(\text{OH})\text{D}_5$  compared to its closest analogues, resulting in  $1\alpha(\text{OH})\text{D}_5$ 's potential for the preventing and treating cancer, was not expected.

B. THE EXAMINER ERRED IN FINDING CLAIM 1, DIRECTED TO  $1\alpha(\text{OH})\text{D}_5$ , OBVIOUS, BECAUSE THE PRIOR ART FAILS TO DISCLOSE OR RENDER OBVIOUS A METHOD FOR MAKING  $1\alpha(\text{OH})\text{D}_5$

The absence of a viable synthetic route or obvious method for making a claimed compound overcomes a presumption of obviousness based on the close relationship between the structures of the claimed compound and the prior art compounds. See In re Hoeksema, 399 F.2d 269, 274-75, 158 USPQ 597, 601 (CCPA 1968). In synthesizing  $1\alpha(\text{OH})\text{D}_5$ , appellants took a naturally occurring starting material and made something that had never been made before. To appellants' knowledge, there was no known method for making  $1\alpha(\text{OH})\text{D}_5$  at the time it was first synthesized by appellants. Therefore, the Examiner's prima facie case of obviousness has been overcome.

In fact, the obvious way of making  $1\alpha(\text{OH})\text{D}_5$  at the time of the invention, if one chose to select a naturally occurring sterol as a starting material, would have been to choose systosterol, which has the same side chain as  $1\alpha(\text{OH})\text{D}_5$ . But this would have been unsuccessful because commercially available systosterol consists of an inseparable mixture of systosterol, campesterol and brassicasterol. The Appellants discovered that, by using commercially available pure stigmasterol instead, they

could synthesize  $1\alpha(\text{OH})\text{D}_5$  with the correct sidechain.

There are many, many analogues of vitamin D2 and vitamin D3. The method by which virtually all of these analogues were made prior to the date of appellants' first synthesis of  $1\alpha(\text{OH})\text{D}_5$  was, almost universally, to functionalize the side chain as a separate chemical moiety and then add the side chain to a truncated vitamin D moiety to obtain the vitamin D analogue. Appellants did not use this conventional technique in synthesizing  $1\alpha(\text{OH})\text{D}_5$ . Uniquely, appellants recognized that a natural compound, stigmasterol, existed, that already had an ethyl group in the C24 position, and that stigmasterol had this ethyl group in one and only one configuration. Appellants recognized that they could prepare an active vitamin D analogue ( $1\alpha(\text{OH})\text{D}_5$ ) without adding a side chain in a separate step to achieve the desired configuration, but rather by taking a naturally occurring compound and using it as a starting material.

Thus, appellants did not use known methodology for making  $1\alpha(\text{OH})\text{D}_5$  with an ethyl group as the functional side chain. Appellants made  $1\alpha(\text{OH})\text{D}_5$  in a totally different way. This method was neither disclosed nor obvious at the time of appellants' invention. Even if the literature contained some motivation or suggestion that  $1\alpha(\text{OH})\text{D}_5$  would have the desirable combination of properties it has, which appellants do not concede, no one had thought to use the naturally occurring sterol, stigmasterol, as starting material, to obtain  $1\alpha(\text{OH})\text{D}_5$ . For this additional reason, the rejection of claim 1 should be reversed.

C. SECONDARY CONSIDERATIONS

Developing an effective antiproliferative compound having low calcemic activity (i.e. low toxicity) has been a goal of the pharmaceutical industry for a long time. None of the named inventors in the four cited references, even though the references disclose generic structures that include  $1\alpha(\text{OH})\text{D}_5$ , knew of the significantly lower calcemic activity of  $1\alpha(\text{OH})\text{D}_5$ , or else they would have made the compound, or at least tried to make it. To appellants' knowledge, no one did. No one, including the appellants when they set out to make  $1\alpha(\text{OH})\text{D}_5$  for the first time, anticipated that an ethyl group at the C24 position in  $1\alpha(\text{OH})\text{D}_5$  would have such a favorable effect on the properties of the compound because the sum total of the existing data could not be used in a predictive way.

Before Appellants synthesized  $1\alpha(\text{OH})\text{D}_5$  there had been a long felt need to develop a vitamin D derivative that has antiproliferative activity but has low calcemic activity. Hundreds, if not thousands, of vitamin D derivatives were made with this objective in mind, but have failed to achieve this desired combination of properties. Many of these vitamin D derivatives are species of Bishop's formula I generic compound. Given the large number of compounds included in Bishop formula I and the unpredictability of the properties of these compounds, the examiner's position (that there was a reasonable expectation that  $1\alpha(\text{OH})\text{D}_5$  would be an effective antiproliferative agent and

cell differentiation agent while having low calcemic activity) is unjustified.

In their Amendment After Final Office Action of December 13, 1999, appellants submitted evidence that  $1\alpha(\text{OH})\text{D}_5$  shows great promise to fulfill a long felt but unmet need for an effective chemopreventive compound. The evidence included an editorial published in the February 5, 1997 issue of the highly respected Journal of the National Cancer Institute that stated in part: "A major focus of chemopreventive research in the field of vitamin D and cancer has been to synthesize analogues of  $1\alpha,25-(\text{OH})_2$  Vitamin  $\text{D}_3$  that have prominent antiproliferative effects against cancer cells without resulting in hypercalcemia ... The study by Mehta et al. reported in the same issue of the Journal [regarding  $1\alpha(\text{OH})\text{D}_5$ ] presents an entirely new class of vitamin D compounds (vitamin  $\text{D}_5$ ).". The study by Mehta, one of the present inventors, described the efficacy of  $1\alpha(\text{OH})\text{D}_5$  in preventing mammary Carcinogenesis.

A second article, published in the Nov. 15, 2000 issue of the Journal of the National Cancer Institute and submitted by appellants in their Jan. 30, 2000 submission, provided further evidence of vitamin  $\text{D}_5$ 's chemo preventative promise. Appellants continue to study the efficacy of  $1\alpha(\text{OH})\text{D}_5$  in the prevention and treatment of cancer, particularly breast cancer, with substantial funding in part from the United States Army.

If the Bishop et al. patent, issued June 9, 1998, taught the benefits of  $1\alpha(\text{OH})\text{D}_5$ , then why has no one besides appellants made

and tested it in the ensuing time? The answer is twofold: no one skilled in the art understood Bishop to teach that  $1\alpha(\text{OH})\text{D}_5$  would have such a favorable combination of therapeutic activity and low calcemic activity, and (2) no one, to Appellants' knowledge, had discovered a synthetic route for making  $1\alpha(\text{OH})\text{D}_5$ .

#### **SUMMARY OF APPELLANT'S ARGUMENT**

The examiner's prima facie case of obviousness is based on the fact that the claimed compound is encompassed by a genus disclosed in four prior art patents, and the examiner's belief, based on the Bishop et al. patent, that an ordinary artisan would have the reasonable expectation that any of the species of the genus would have similar properties and, thus, the same use as the genus as a whole. Appellants have rebutted this prima facie case of obviousness with factually supported objective evidence that there was, in fact, no reasonable expectation that  $1\alpha(\text{OH})\text{D}_5$  would have the properties it has. Appellants have also pointed out the absence of prior art suggesting a viable synthetic route for making  $1\alpha(\text{OH})\text{D}_5$ . Finally, appellants have provided evidence that  $1\alpha(\text{OH})\text{D}_5$  shows great promise in fulfilling the long felt but unmet need for a chemopreventive agent having low calcemic activity. A decision reversing the examiner's rejection of claim 1 is respectfully requested.

#### **VI. APPENDICES**

##### **I. Claim on Appeal**

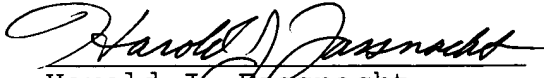
II. References cited by the Examiner.

III. Excerpt from file wrapper of Knutson Patent No.

5,488,120

IV. Declaration of Dr. Robert Moriarty

Respectfully submitted,

  
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Dated: *April 23, 2001*

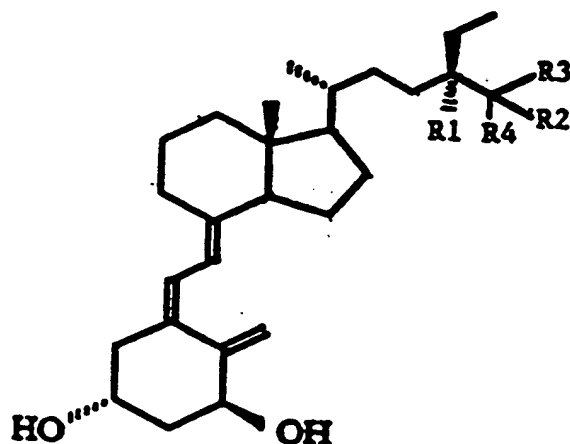
APPENDIX I

PENDING CLAIM

# CLAIMS

We claim as our invention:

1. A compound of formula I:



I

wherein:

- R1 is hydrogen;
- R2 is -CH<sub>3</sub>;
- R3 is -CH<sub>3</sub>; and
- R4 is hydrogen.

**APPENDIX II**

**ART CITED BY EXAMINER**

# United States Patent [19]

Holick et al.

[11] Patent Number: 4,728,643

[45] Date of Patent: Mar. 1, 1988

## [54] METHOD OF TREATING PSORIASIS

[75] Inventors: Michael F. Holick, Sudbury; Julia McLaughlin, W. Roxbury, both of Mass.

[73] Assignee: The General Hospital Corporation, Boston, Mass.

[21] Appl. No.: 667,813

[22] Filed: Nov. 2, 1984

[51] Int. Cl.<sup>4</sup> ..... A61K 31/59

[52] U.S. Cl. .... 514/167; 514/863

[58] Field of Search ..... 514/167

## [56] References Cited

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4,230,701 10/1980 Holick et al. .... 514/167  
4,391,802 7/1983 Suda et al. .... 514/167  
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Honma, Y. et al., Proceedings of the National Academy of Sciences, USA, 80:201 (1983).

Shiina, Y., et al., Archives of Biochemistry and Biophysics, 220:90 (1983).

Primary Examiner—Leonard Schenkman

Assistant Examiner—Joseph A. Lipovsky

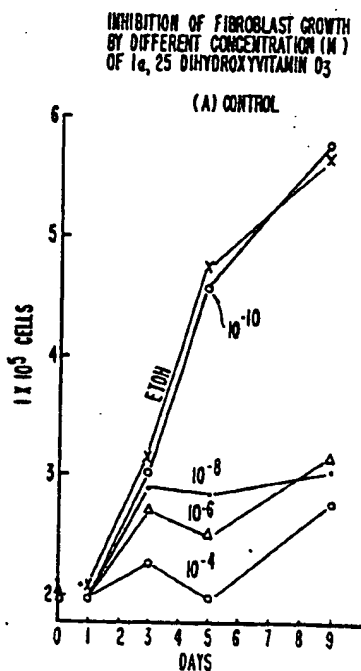
Attorney, Agent, or Firm—Saidman, Sterne, Kessler & Goldstein

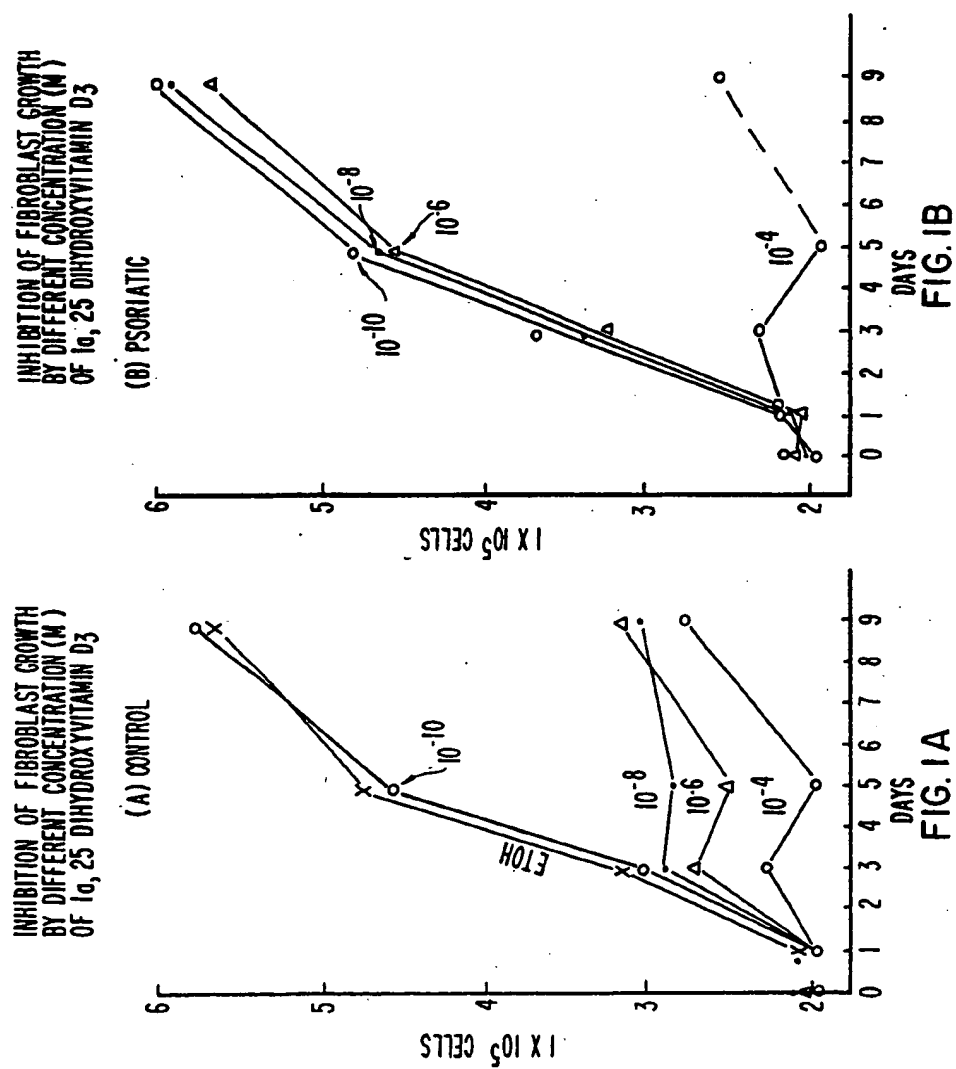
## [57]

## ABSTRACT

A method of treating psoriasis in a patient which comprises administering to said patient an effective amount of a vitamin D compound which is capable of stimulating the differentiation of cultured tumor cells or normal rodent or human fibroblasts or keratinocytes in vitro.

15 Claims, 2 Drawing Figures





## METHOD OF TREATING PSORIASIS

## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The present invention relates to a novel method of treating psoriasis, a disease of the skin. The method comprises using vitamin D-related compounds.

## 2. Brief Description of the Background Art

Psoriasis is a disease of the epidermis and a major cause of disability and disfigurement, for between 2,000,000 and 8,000,000 persons in the United States. Of these, about 100,000 are severely affected.

The disease is diagnosed by the presence of scaling erythematous on the scalp and extender aspects of the arms and legs; psoriatic lesions often are accentuated on the sites of repeated trauma, such as the elbows and knees. The papules or plaques of psoriasis often contain a silvery-white micaceous scale that is relatively easily removed in layers. There is a several fold increase in the normal number of the basal cells of the epidermis. This increase in the basal cell population reduces the turnover time of the epidermis from the normal 27 days to 3-4 days. This shortened interval leads to the consequence that normal events of cell maturation or keratinization do not occur, and this failure of maturation is reflected by an array of abnormal morphologic and biochemical changes. Numerous cytologic, histologic, histochemical and biochemical alterations are now known to be the result, rather than the cause of the disease process. The only main fact known at this time about the fundamental cause of psoriasis is that the predisposition to its development is genetically transmitted. (This introduction is basically taken from Harrison's *Principles of Internal Medicine*, 10th Ed., Vol. 1, pp. 256 and 257).

The treatment of psoriasis still remains the province of dermatologists. The most effective treatment in the control of localized psoriasis for most patients is the topical use of corticosteroids with a plastic wrap and ultraviolet light or sunlight exposures. On certain patients who have generalized psoriasis, it has been necessary to use a variety of systemic chemotherapeutic agents, especially methotrexate; the latter has the capacity to inhibit cell replication without a proportionate inhibition of cell function; i.e. keratinization. Photochemotherapy was introduced in 1974, in the so-called PUVA treatment. In this treatment, psoralen is administered two hours before total body irradiation with a special light system that emits predominantly long wave ultraviolet light. The light alone is ineffective in producing erythema or remission of psoriatic lesions; however, in the presence of one of the psoralens, the UV-A light becomes a potent photoactive agent and produces a remission of psoriatic lesions after several exposures. Photochemotherapy requires specialized knowledge and lighting systems delivering precisely measured amounts of ultraviolet light.

Along quite different areas of research, Holick et al. (*New England Journal of Medicine*, 303: 349-354 (1980)) have studied the feasibility of using the skin as the organ for the synthesis and absorption of vitamin D metabolites. These investigators demonstrated that topical application of various vitamin D metabolites or provitamin forms followed by phototherapy results in elevated serum levels of dihydroxy-vitamin D<sub>3</sub>. It was therefore suggested that topical application of vitamin D analogues may be an effective method of therapy for

diseases involving calcium, phosphorus and bone metabolism problems. It is only recently, however, that it has become clear that the skin itself may be a target tissue for 1,25-(OH)<sub>2</sub>-D<sub>3</sub> (Stumpf, W. E. et al., *Science*, 206:1188-1190 (1979)). Cells isolated from the skin of rats, mice, and humans, and from cultured human skin fibroblasts and keratinocytes contain a high affinity (1.0×10<sup>-10</sup> M) low capacity receptor-like protein for 1,25-dihydroxy-vitamin D<sub>3</sub> (Franceschi, et al., *Arch. Biochem. Biophys.* 210: 1-13 (1979); Simpson, R. U. et al., *P.N.A.S. USA*, 77: 5822 (1980); Colston, K. et al., *Endocrinology*, 107: 1916 (1980); Feldman D. et al., *Journal of Clinical Endocrinology & Metabolism*, 51: 1463 (1980); Eil, C. et al., *P.N.A.S. USA*, 78: 2562 (1981); and Clemens, T. L. et al., *J. Clin. Endocr. Metab.* 56: Apr. 1983). A specific biological function for 1,25-(OH)<sub>2</sub>-vitamin D<sub>3</sub> in the skin, however, has yet to be discovered. Nevertheless, evidence has come forth supporting the concept that the dihydroxy metabolite of the vitamin does have biologic actions in the skin. This was accomplished by evaluating the biological activity of 1,25-dihydroxy-D<sub>3</sub> simultaneously in cultured human skin fibroblasts that either possessed or lacked a cytosolic receptor-like protein for the hormone (Clemens, T. L. et al., *J. Clin. Endocrinol. Metab.*, 56: Apr. 1983). The receptor-negative skin fibroblasts were obtained from a patient with a rare bone disorder called vitamin D dependent rickets, type ii, a heritable disorder caused by a defective or complete absence of a cytoplasmic or nuclear receptor for 1,25-dihydroxy vitamin D. The dihydroxy metabolite of vitamin D<sub>3</sub> caused a dose-dependent inhibition of cell growth in receptor positive skin fibroblasts (about 40-50% reduction in cell growth was observed in cultures containing 10<sup>-6</sup> and 10<sup>-8</sup> M of hormone and 12% in cultures containing 10<sup>-10</sup> M of 1,25-(OH)<sub>2</sub>-D<sub>3</sub>), and, by contrast, had absolutely no effect on the growth of receptor negative skin fibroblasts.

The aforementioned seemingly two divergent lines of research, the treatment of psoriasis on the one hand, and the effects of vitamin D<sub>3</sub> on skin components on the other, remained heretofore unrelated until, by the present invention, they have been brought together.

## SUMMARY OF THE INVENTION

This invention arose out of the initial observation that when psoriatic cells were incubated in vitro with 1,25-(OH)<sub>2</sub>-D<sub>3</sub> at physiologic concentrations, they were resistant to growth inhibition effects, whereas at pharmacologic concentrations (10<sup>-6</sup> and 10<sup>-4</sup> M), the dihydroxy metabolites of vitamin D<sub>3</sub> was capable of inhibiting the cells growth of these psoriatic fibroblasts. Thus vitamin D, as well as its homologues, analogues and hydroxylated metabolites, can be utilized effectively in the treatment of psoriasis.

An accurate correlation between an in vitro test or tests and antipsoriatic treatment in vivo has further been established. According to this correlation, the vitamin D compounds usable in the treatment of psoriasis are those capable of stimulating or inducing the differentiation of tumor or normal cell lines which possess receptors for 1,25-dihydroxyvitamin D<sub>3</sub>. Normal cell lines include cultured rodent and human keratinocytes. Active compounds are also those capable of increasing the enzymatic activity of transglutaminase in the same cell system, or are those capable of inhibiting the cell

growth in vitro of human skin fibroblasts. Details of these tests can be found below.

The vitamin D compounds, homologues, analogues or metabolites thereof which are useful in treating psoriasis are those which demonstrate activity in any of the in vitro tests.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Generally, an active compound is one which induces differential at physiologic concentration of a tumor or a normal cell line which possess receptors for 1,25-dihydroxyvitamin D<sub>3</sub>. Among normal useable lines are for example human or rodent keratinocytes or fibroblasts. Among tumor lines are HL-60 cell line, M-1 cell line, breast tumor cells. A few tests will be described in further detail herein.

A first test is one which measures the differentiation of cultured keratinocytes. The assay is essentially the one described by Hosomi, et al., "Regulation of Terminal Differentiation of Cultured Mouse Epidermal Cells by 1 $\alpha$ ,25-dihydroxy Vitamin D<sub>3</sub>," *Endocrinology*, 113: 1950 (1983) for mice, or that described by Clements et al. supra for the human system, both herein incorporated by reference. Briefly, epidermal cells are prepared from newborn C57BL mice by overnight treatment with trypsin at 4° C. followed by separation of the epidermis from the dermis with forceps. Cells are plated at a density of 10<sup>6</sup> cells per 4.5 cm<sup>2</sup> well and grown in Eagle minimum essential medium (MEM) (supplemented with 10% fetal calf serum (FCS)). Cells can also be grown in low calcium medium, Eagle MEM, without calcium supplemented with 10% dialyzed FCS. Calcium concentration of the low calcium medium can be from about 0.01–0.5 mM, whereas a conventional MEM plus 10% FCS usually may contain 1.0–2.0 mM calcium. Cells are incubated in a humidified CO<sub>2</sub> incubator at 37° C. All experiments are performed on the primary cultures. Twenty-four hours after plating, the medium is changed and the vitamin D compound is added at concentration of 0.12, 1.2 and 12 nM (0.05, 0.5 and 5.0 mg/ml, respectively). Control cultures are supplemented with ethanol at a final concentration of 0.5%. The media with and without the vitamin D is renewed every 3–4 days. (FCS contains 1,25-(OH)<sub>2</sub>-D<sub>3</sub> at 0.12 nM (Tanaka, H. et al., *Biochem. J.*, 204: 713 (1982)). Therefore, the endogenous concentration of the vitamin in the control culture medium which contains 10% FCS is negligible.)

Differentiation of epidermal cells in culture is examined morphologically by

- (1) counting the number of squamous and enucleated cells sloughed off into the medium,
- (2) counting the number of squamous and basal cells attached to the dishes,
- (3) formation of a cornified envelope,
- (4) the cell size and cell density, or
- (5) morphological changes seen under a light microscope, or some or all of the above in combination.

Floating cells are collected from the medium. Then the cultures are washed with phosphate buffered saline (PBS) and attached cells are dissociated by treatment with 0.05% trypsin and 0.1% EDTA solution at 37° C. for 20–30 min. Cell suspensions are then divided into two portions: one for counting the numbers of squamous and basal cells and the other for counting cornified envelopes. Since basal cells are small and round, whereas squamous and enucleated cells are large and

flat, they are readily distinguishable in a hemocytometer. The method of Sun and Green (Cell, 9: 511 (1976)) can be used to determine the presence of a cornified envelope. The cells are resuspended in 10 mM Tris-HCl (pH 7.4) containing 1% beta-mercaptoethanol and 1% sodium dodecylsulfate at a density of 5:30×10<sup>6</sup> cells/ml. The mixture stands for 10 minutes at room temperature and then insoluble cells are counted in a hemocytometer under a phase contrast microscope.

The size of cells can be measured in photographs with a stage micrometer as a standard. The density distribution of cells is measured by density gradient centrifugation in Percoll®. Epidermal cells 8–11×10<sup>6</sup>/ml are suspended in PBS containing 40% Percoll, placed in a 10 ml polycarbonate tube, and centrifuged at 15,000×g at 3° C. for 30 minutes in an angled rotor. Fractions are collected by use of density marker beads. For light microscopic observation, cells grown in a glass cover slip are fixed with either 10% formalin or methanol/acetic acid (3:1) and stained with hemotoxiline and cothine or rhodanile blue.

In the presence of an active vitamin D compound useful for psoriatic treatment, differentiation of epidermal cells is markedly stimulated. Focal stratification is formed in places on top of the epidermal cell sheets. Stratified foci increase in number and size and contiguous foci coalesce. In the uppermost layer of stratified foci, cells produce an amorphous material staining red with hemotoxiline and cothine and rhodanile blue. Some cells are enucleated and some have a thick pyknotic nucleus. Differentiated cells slough off into the medium so that the total number of cells attached to the dish decrease continuously with the time of cultivation. The fraction of attached basal cells decrease sharply in the presence of an active vitamin D compound. For example, close to 100% of the cells are basal cells on day 0, but only about 25% on day 3 and less than 10% after day 10. In a control culture on the other hand, more than 60% of the cells are basal cells during the first six days and usually 30–40% or so remain basal on day 10. of squamous cells increases in the vitamin D active treated cultures, first among the attached cell population and then among the sloughed off floating cells.

Epidermal differentiation can be quantified by counting cornified envelopes remaining after cell lysis with a solution containing 1% sodium dodecylsulfate and 1% beta-mercaptoethanol. When the cells are grown in the presence of 12 nM active vitamin D compound, the percentage of cells with a cornified envelope increases with time of cultivation. The percentage is greatest after 10 days in culture when about 60–70% of the cells have an envelope. In contrast, the percentage of control cultures remain at 20% or less during a two week observation period.

The cells obtained in the presence of an active vitamin D compound for 3 days are larger and lighter than those in its absence. The diameter of cells in the treated cultures is usually about 25±10  $\mu$ m, compared with about 17±5  $\mu$ m in a control.

Cell density by Percoll gradient centrifugation indicates that, when grown in the presence of an active vitamin D compound for 3 days, about 65% of the cells are collected in the lightest fraction with a density of about 1.017–1.027, whereas about 40% of the control cells are recovered in this fraction. Concomitantly, the number of cells in a heavier fraction (density) between about 1.06 and 1.08 decrease in the treated cultures.

Similar results are obtained at day 7. Human keratinocytes can be grown by the method of Clemens et al., supra, and analyzed in an identical manner.

A second test is that of inhibition of human skin fibroblasts. This test is found in Clemens et al., *J. Clin. Endocr. Metab.*, 56: 824 (1983), herein incorporated by reference. Briefly, skin cells are isolated from surgically obtained normal human skin from mammary, face, thigh, etc. of a normal patient.

Normal skin biopsies are placed immediately in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin G (75 U/ml), and streptomycin (50 ng/ml). After removal of subcutaneous fat and the deep reticular layer of the dermis, the tissues are minced and placed in 10 ml 0.25% trypsin at 4° C. overnight.

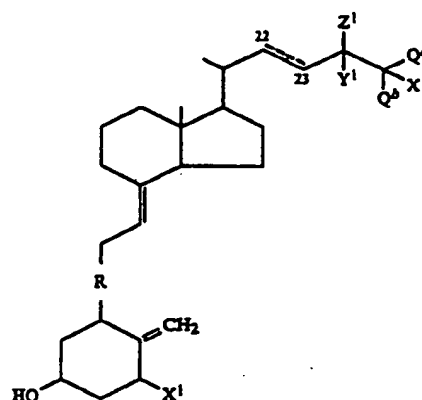
Fibroblasts are plated at  $7-10 \times 10^4$  cells in 35-mm Costar dishes in DMEM containing 5% NBS. After attachment of cells (6 hours), the media are aspirated and replaced with fresh medium containing ethanol alone (0.01%) or ethanol (0.01%) containing compound at  $10^{-10}$ ,  $10^{-8}$ ,  $10^{-6}$ , or  $10^{-4}$  M. At intervals thereafter, cells are harvested from duplicate plates by trypsinization and counted in a Coulter counter. Control and compound supplemented media are replaced at 4-day intervals. Normal foreskin fibroblasts, plated at  $5 \times 10^4$  cells/well (DMEM; 5% NBS), can also be treated with ethanol (0.01%) alone or ethanol containing compound ( $10^{-10}$ - $10^{-4}$  M). After 4 days, fresh medium containing the appropriate sterol is replaced, and cells are counted 2 days later, 6 days after plating.

An alternative and perhaps faster and more accurate test of correlation for active vitamin D compounds is the in vitro activity of transglutaminase, in the keratinocyte culture. The enzymatic test is carried out according to standard transglutaminase assays, Scott, K. F. F. et al., *J. Cell. Physiol.* 111:111-116 (1982). Any compound which when present at a concentration of  $10^{-12}$  M to  $10^{-3}$  M increases the enzymatic activity by 25% or more, preferably 50% or more, most preferably 100% or more is considered an active compound.

Use of the HL-60 cells in an in vitro test is described in Shiina, et al., *Arch. Biochem. Biophys.* 220:90 (1983). Use of the M1 cells in an in vitro test is described in Honma et al., *PNAS, USA* 80:201-204 (1983). Both of these references are herein incorporated by reference.

Any vitamin D compound which at in vitro concentrations of  $10^{-12}$  M to  $10^{-3}$  M is capable of cellular differentiation or inhibiting of fibroblast growth by at least 25%, preferably 50% is considered active.

Among the preferred compounds usable in the present invention are those of the formula (I):



wherein

the bond between carbons C-22 and C-23 is single or

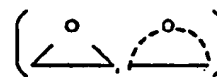
double; Y<sup>1</sup> is hydrogen, F, —CH<sub>3</sub> or —CH<sub>2</sub>CH<sub>3</sub>;

Z<sup>1</sup> is F, H or X<sup>1</sup>;

Q<sup>a</sup> is CF<sub>3</sub> or CH<sub>2</sub>X<sup>1</sup>;

Q<sup>b</sup> is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy



group;

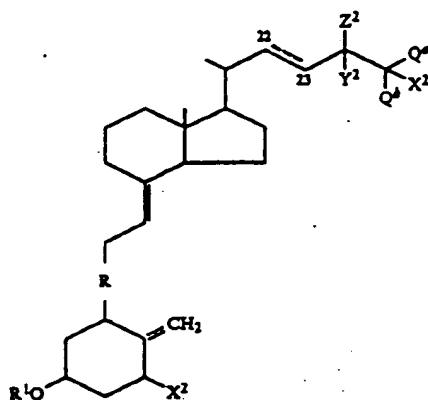
wherein X is selected from the group consisting of hydrogen and —OH.

When the compounds of formula (I) have a double bond at position C-22, they are derivatives of vitamin D<sub>2</sub>, whereas if the bond at that position is single, and there is a lack of C<sub>24</sub> alkyl, they are derivatives of vitamin D<sub>3</sub>. The latter are preferred.

Preferred are those compounds derived from vitamins D<sub>3</sub> or D<sub>2</sub>; 1-hydroxy-vitamins D<sub>3</sub> or D<sub>2</sub>; 1,25-dihydroxy vitamins D<sub>3</sub> and D<sub>2</sub>; 24,25-dihydroxy vitamins D<sub>3</sub> or D<sub>2</sub>; 25,26-dihydroxy vitamins D<sub>3</sub> or D<sub>2</sub>; 1,24,25-trihydroxy vitamins D<sub>3</sub> or D<sub>2</sub>. Most preferred among these are vitamins D<sub>3</sub> or D<sub>2</sub>; 1-hydroxy-vitamins D<sub>3</sub> or D<sub>2</sub>; and 1,25-dihydroxy-vitamins D<sub>3</sub> or D<sub>2</sub>, especially 5,6-epoxy derivatives of vitamin D and its metabolites, as well as the side chain fluoro derivatives of 1, 25 (OH)<sub>2</sub> vitamin D and 1α (OH) vitamin D.

Among other preferred compounds are water soluble derivatives of the aforementioned compounds of formula (I) obtained by solubilizing such compounds by attaching thereto glycosidic residues such as those disclosed in Holick, U.S. Pat. No. 4,410,515. Alternative methods of solubilization are by conjugating compounds of formula (I) to glycosyl orthoester residues, as disclosed in copending U.S. Ser. No. 607,117 by Holick et. al., filed May 3, 1984. The disclosures of the aforementioned patent and application are herein incorporated by reference and made a part hereof.

Of interest are compounds of the formula (II):



wherein

Y<sup>2</sup> is hydrogen, fluorine, methyl or ethyl;

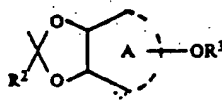
Z<sup>2</sup> is F, H or X<sup>2</sup>

Q<sup>4</sup> and Q<sup>4'</sup> have the same meanings as in formula (I);

R is a double bond or an epoxy group;

X<sup>2</sup> is selected from the group consisting of hydrogen, 25 and OR<sup>1</sup>,

where R<sup>1</sup> is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or R<sup>1</sup> is an orthoester glycoside moiety of the formula (III).



where

A represents a glucofuranosyl or glucopyranosyl ring;

R<sup>2</sup> is hydrogen, lower alkyl, aralkyl, or aryl; and

R<sup>3</sup> is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, with the proviso that at least one of the R<sup>1</sup> is either a glycosidic residue or an orthoester glycoside moiety.

The vitamin D compounds are prepared or obtained according to the disclosures of the aforementioned references. In particular, the 5,6-epoxy derivatives of vitamin D<sub>3</sub> are obtained as described in *Jpn. Kokai Tokkyo Koho JP 58,216,178* [83,216,178], Dec. 15, 1983.

The fluoro derivatives are made or obtained as described in Shiina, et al., *Arch. Biochem. Biophys.* 220:90 (1983).

The compounds of the invention can be administered 55 in any appropriate pharmacological carrier for oral, parenteral, or topical administration. They can be administered by any means that effects palliating conditions of psoriasis in humans. The dosage administered will be dependent upon the age, health and weight of the recipient; kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired. Generally, systemic daily dosage of active ingredient compounds will be from about 0.001 micrograms/kg to 100 micrograms/kg preferably 0.1 to 1.0 micrograms 60 per kg of body weight. Normally, from 0.1 to 100 micrograms/kg per day, in one or more applications per day is effective to obtain the desired results. Topical

dosage would be 0.001 micrograms to 100 micrograms/cm<sup>2</sup> area of skin.

The compounds can be employed in dosage forms such as tablets, capsules, powder packets, or liquid solutions, suspensions or elixirs for oral administration, sterile liquid for formulations such as solutions or suspensions for parenteral use. Alternatively, the compounds can be present in a pharmacologically inert topical carrier such as one comprising a gel, an ointment or a cream, including such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters or mineral oils. Other possible carriers are liquid petrolatum, isopropylpalmitate, polyethylene glycol ethanol 95%, polyoxyethylene monolaurate 5% 15 in water, sodium lauryl sulfate 5% in water, and the like. Materials such as anti-oxidants, humectants, viscosity stabilizers and the like may be added, if necessary.

The compounds can also be administered by means of pumps or tapes.

20 Having now generally described this invention, the same will be understood by reference to an example which is provided herein for purposes of illustration only and is not intending to be limited unless otherwise specified.

#### EXAMPLE

Skin biopsies from involved and uninvolved sites were obtained from psoriatic patients and therefrom were obtained cultured fibroblasts. An analysis of cultured fibroblasts from the psoriatic patients revealed that these possess high affinity low capacity receptors for 1,25-(OH)<sub>2</sub>-D<sub>3</sub> and that the K<sub>d</sub> and density for these receptors in fibroblasts from the uninvolved areas were essentially no different from that found in cultured 30 skin fibroblasts from normal subjects. In addition, the fibroblasts from involved sites possessed receptors for 1,25-dihydroxyvitamin D<sub>3</sub> that have a normal affinity constant but possibly as much as 100% decrease in number of receptor sites when compared to the uninvolved fibroblasts.

It was next determined if cultured fibroblasts from psoriatic patients would respond to 1,25-dihydroxyvitamin D<sub>3</sub> by causing an inhibition of cell growth. Cultured human fibroblasts from normal and psoriatic subjects were incubated with either no 1,25-dihydroxyvitamin D<sub>3</sub> or 1,25-dihydroxy vitamin D<sub>3</sub> at either 10<sup>-10</sup>, 10<sup>-8</sup>, 10<sup>-6</sup>, or 10<sup>-4</sup> M. Fibroblasts from the normal subjects responded as expected in a dose dependent manner. However, none of the fibroblasts obtained from six different subjects with psoriasis responded to 1,25-dihydroxyvitamin D<sub>3</sub> at 10<sup>-8</sup> M in a similar fashion as the controls. When psoriatic cells were incubated with 1,25-(OH)<sub>2</sub>-D<sub>3</sub> at 10<sup>-6</sup> M, there was a small but significant effect on inhibiting cell growth in some of the subjects studied (who were resistant to up to 10<sup>-6</sup> M of 1,25-dihydroxyvitamin D<sub>3</sub>). In one subject, a detailed time course and dose response revealed a very small response at 10<sup>-6</sup> M while 1,25-dihydroxy vitamin D<sub>3</sub> at 10<sup>-4</sup> M was very effective in inhibiting cell growth (FIG. 1).

What is claimed as new and desired to be covered by U.S. Letters Patent is:

1. A method of treating the disease of psoriasis in a patient affected by said disease which comprises administering to said patient by oral or parenteral means an effective amount of a vitamin D compound, which compound when tested in vitro is capable of stimulating the differentiation of cultured tumor cells.

2. The method of claim 1 wherein said tumor cells are human cells.

3. The method of claim 1 wherein said tumor cells are HL-60 cells or M-1 cells.

4. A method of treating the disease of psoriasis in a patient affected by said disease which comprises administering to said patient by oral or parenteral means an effective amount of a vitamin D compound, which compound when tested in vitro is capable of stimulating the differentiation of cultured normal rodent or human keratinocytes or fibroblasts.

5. A method of treating the disease of psoriasis in a patient affected by said disease which comprises administering to said patient by oral or parenteral means an effective amount of a vitamin D compound, which compound when tested in vitro is capable of inhibiting normal fibroblast cell growth.

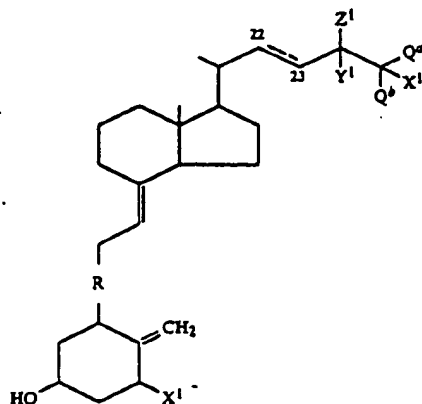
6. The method of claim 3, wherein said fibroblasts are human fibroblasts.

7. A method of treating the disease of psoriasis in a patient affected by said disease which comprises administering to said patient by oral or parenteral means an effective amount of a vitamin D compound, which compound when tested in vitro is capable of increasing the enzymatic activity of transglutaminase in cultured keratinocytes.

8. The method of any of claims 1-7, wherein said vitamin D compound is selected from the group consisting of 1,25-dihydroxyvitamin D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>2</sub>, 1-hydroxyvitamin D<sub>3</sub>, and 1-hydroxyvitamin D<sub>2</sub>.

9. The method of claim 8 wherein said vitamin D compound is 1,25-dihydroxyvitamin D<sub>3</sub>.

10. A method of treating the disease of psoriasis in a patient affected by said disease which comprises administering to said patient by oral or parenteral means an effective amount of a vitamin D compound, which compound when tested in vitro is capable of stimulating the differentiation of a cultured cell said cell selected from the group consisting of (a) a cultured tumor cell and (b) a cultured normal rodent or human, keratinocyte or fibroblast cell; said vitamin D compound having the formula (I):

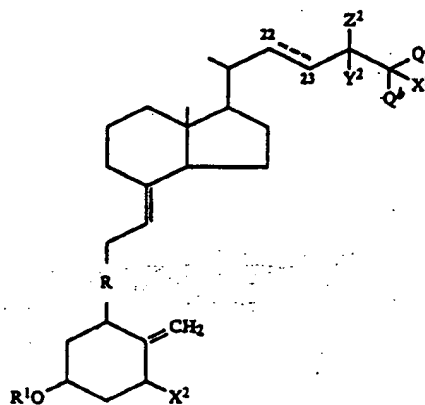


wherein

the bond between carbons C-22 and C-23 is a single or double bond; Y¹ is hydrogen, F, CH<sub>3</sub> or CH<sub>2</sub>CH<sub>3</sub>; Z¹ is F, H or X¹; Qᵃ is CF<sub>3</sub> or CH<sub>2</sub>X¹; Qᵇ is CF<sub>3</sub> or CH<sub>3</sub>; R is a double bond or an epoxy group;

wherein X¹ is selected from the group consisting of hydrogen and OH.

11. A method of treating the disease of psoriasis in a patient affected by said disease which comprises administering to said patient by oral or parenteral means an effective amount of a vitamin D compound, which compound when tested in vitro is capable of stimulating the differentiation of a cultured cell, said cell selected from the group consisting of (a) a cultured tumor cell and (b) a cultured normal rodent or human keratinocyte or fibroblast cell; said vitamin D compound having the formula (II):



wherein

the bond between carbons C-22 and C-23 is a single or double bond; Y² is hydrogen, fluorine, methyl, or ethyl;

Z² is F, H or X²

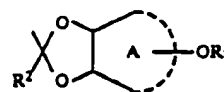
Qᵃ is CF<sub>3</sub> or CH<sub>2</sub>X²;

Qᵇ is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

X² is selected from the group consisting of hydrogen, and OR¹,

wherein R¹ is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or R¹ is an orthoester glycoside moiety of the formula (III):



where

A represents a glycofuranosyl or glucopyranosyl ring;

R² is hydrogen, lower alkyl, aralkyl, or aryl; and

R³ is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, with the proviso that at least one of the R¹ is either a glycosidic residue or an orthoester glycoside moiety.

12. The method of any of claims 10-11 wherein said cultured cell (a) is a human tumor cell.

13. The method of any of claims 10-11 wherein said cultured cell (a) is an HL-60 cell or an M-1 cell.

14. The method of any of claim 10-11 wherein said vitamin D compound is capable, when tested in vitro, or inhibiting the growth of normal fibroblast growth.

15. The method of claim 14 wherein said fibroblasts are human fibroblasts.

# United States Patent [19]

Holick et al.

US005254538A

[11] Patent Number: 5,254,538

[45] Date of Patent: Oct. 19, 1993

[54] METHOD OF TREATING PERIODONTAL DISEASE

[75] Inventors: Michael F. Holick, Sudbury; Xiao Tian, Boston, both of Mass.

[73] Assignee: Trustees of Boston University, Boston, Mass.

[21] Appl. No.: 826,230

[22] Filed: Jan. 27, 1992

## Related U.S. Application Data

[63] Continuation of Ser. No. 416,781, Oct. 4, 1989, abandoned.

[51] Int. Cl.<sup>5</sup> ..... A61K 31/70; A61K 31/59; A61K 7/16

[52] U.S. Cl. .... 514/35; 514/167; 424/49

[58] Field of Search ..... 514/35, 167, 928; 424/49

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Primary Examiner—Frederick E. Waddell

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## [57] ABSTRACT

The invention relates to methods for enhancing wound healing; enhancing gastric, duodenal, esophageal, decubitus, genito urinary ulcer and ulcerative keratitis healing; inhibiting scar formation; and treating periodontal disease in an animal by the topical, oral parenteral, transdermal or ophthalmic administration of a vitamin D compound.

8 Claims, 2 Drawing Sheets

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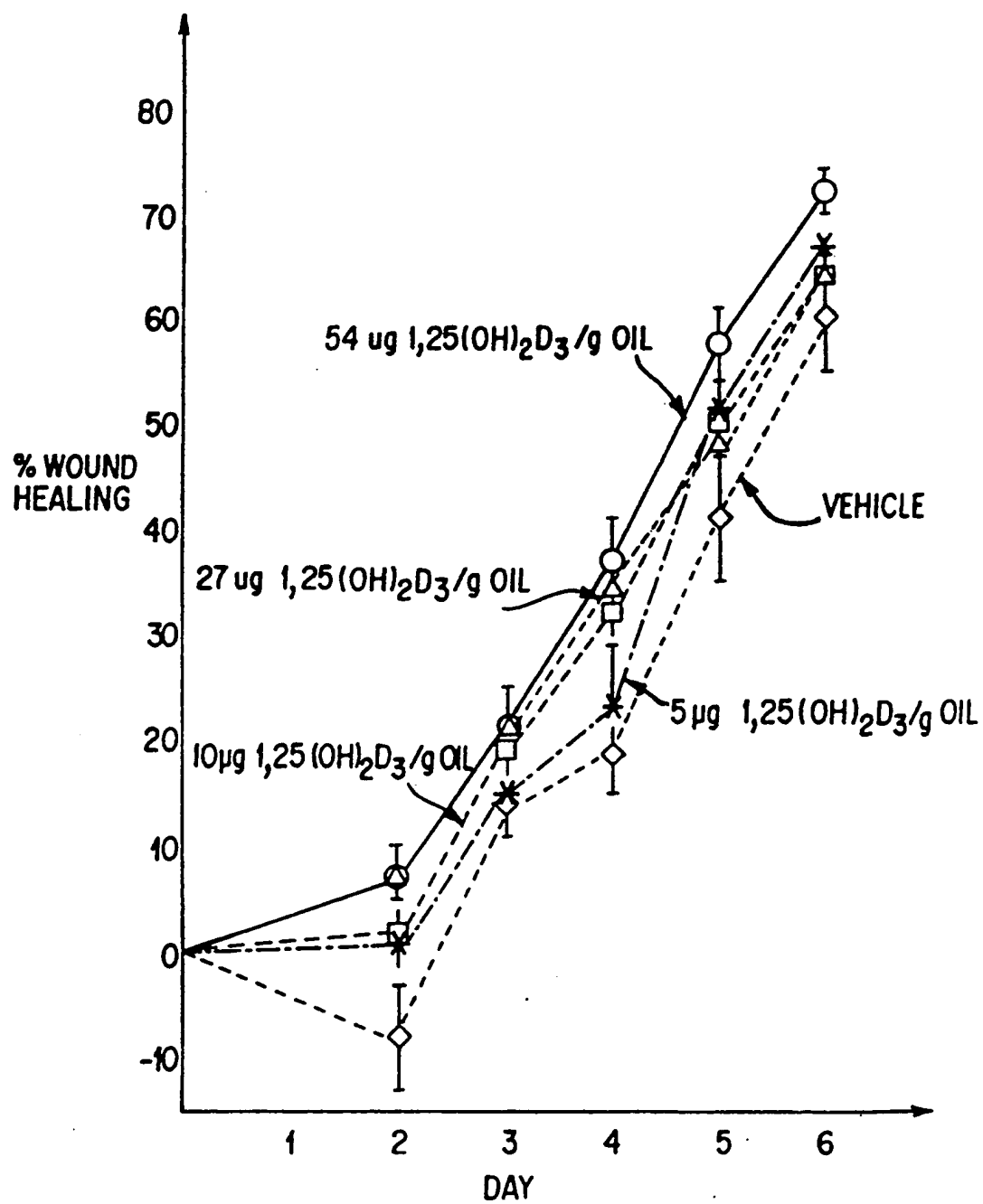


FIG. 1

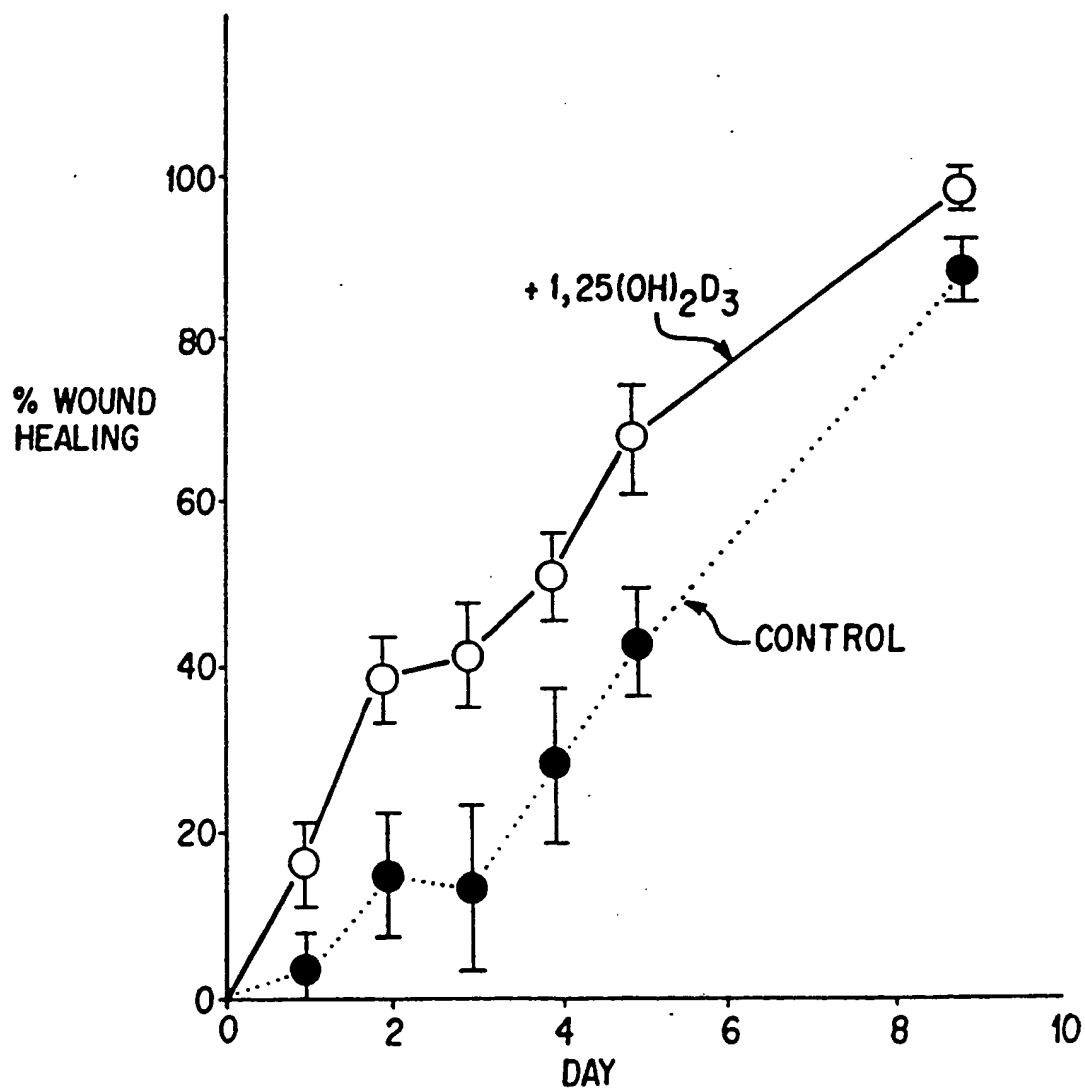


FIG. 2

## METHOD OF TREATING PERIODONTAL DISEASE

This application is a continuation of application Ser. No. 07/416,781, filed Oct. 4, 1989 now abandoned.

### FIELD OF THE INVENTION

The invention is in the field of medicinal chemistry. In particular, the present invention relates to novel methods for accelerating wound and ulcer healing and for treating periodontal disease using vitamin D-related compounds.

### BRIEF DESCRIPTION OF THE BACKGROUND ART

Human skin is a complex integration of different types of cells and tissues which form an organ. Skin is also the primary seat of the sense of touch and creates a covering for the protection of the deeper tissues. The skin also plays an important role in the regulation of body temperature and is also an excretory and absorbing organ. Skin consists primarily of a layer of vascular tissue and an external covering of epithelium known as the epidermis. On the surface layer are the sensitive papillae and alongside or imbedded beneath it are certain specialized organs, specifically the sweat glands, hair follicles, and sebaceous glands.

In order to defend the tissues below from trauma, the skin must be tough, flexible, and highly elastic. As a result of this function, injuries to the skin can occur. Wounds, which are caused by physical means, result in a disruption of the normal continuity of the structures of the skin. Examples of wounds include cuts, punctures, lacerations, etc.

Whereas skin is composed of an external covering of epithelium, the stomach lining is also composed of internal epithelium (endothelium). Gastric ulcers are a result of damage or erosion of the stomach lining. Gastric ulcers occur along the lesser curvature of the stomach where the pyloric glands border the oxyntic gland. They are usually 1 to 2.5 cm in diameter; however, they can vary from a few mm to several cm. Ulcers are usually round, oval or elliptical, with sharply defined margins. The surrounding mucosa is often hyperemic and edematous. Ulcers penetrate into the submucosa or muscular layer. A thin layer of gray or white exudate usually covers the base of the ulcers; this layer is composed of fibrinoid, granulation and fibrous tissue layers. During healing, fibrous tissue in the base contracts the ulcer and may distort the surrounding tissue. Healing continues as granulation tissue fills the base and epithelium from the ulcer edges cover its surface.

Healing usually requires two to six weeks but may require a longer time, especially if the ulcer is large or of a longstanding nature. If complete healing of the ulcer does not occur (as monitored by X-ray or endoscopic exam), surgery is usually considered in an effort to prevent complications or a prolonged, distressing course. *The Merck Manual of Diagnosis and Therapy*, 14th ed., by Merck Sharp & Dohme Research Laboratories (1982).

The mechanism of epithelial wound healing is a complex process involving ultrastructural changes of epithelial cells. These changes allow for detachment from neighboring cells, migration and subsequent reattachment. The migration of epithelial cells has been found to depend on a suitable matrix composed of fibrin, fibro-

nectin or basement membrane which traverse the wound. Clark, R., *J. Am. Acad. Derm.*, 13:701-718 (1985); Zitelli, J., *Adv. Dermatol.* 2:243-268 (1987).

There are two types of healing processes: (1) primary union or first intention healing and (2) secondary union or second intention healing. Primary union occurs when a clean wound with a minimal loss of tissue heals together cleanly. The process involves clotting and formation of a crust or scab to seal the wound; an acute inflammatory reaction; reepithelialization of the surface and fibrous bridging due to fibrin followed by complete sealing of the wound by an epithelial covering. Thereafter, hair follicles, sebaceous glands and sweat glands may subsequently regenerate. The process of second intention healing requires the removal of necrotic debris. The gap in the wound then fills in with fibrous materials.

When dealing with gastric ulcers the major objectives of therapy are relief of pain and healing of the ulcer. In a number of countries (not the U.S.) carbenoxolene is used to treat gastric ulcers. Carbenoxolene is a hydrolytic product of glycyrrhizic acid (derivative of licorice); it has been shown to increase the rate of gastric ulcer healing. Braunwald, E., *Harrison's Principles of Internal Medicine* 11th ed. p. 1247. It appears to increase the life span of gastric mucosal epithelial cells and increase the secretion and viscosity of gastric mucus. However, carbenoxolene has aldosterone-like effects, therefore it tends to increase the rate at which the body retains sodium and water. These effects may be blocked by aldosterone-antagonists, however the antagonists obliterate the healing effects of the carbenoxolene. There is a need for therapies which can promote healing without the negative side effects.

It has recently become clear that the skin may be a target tissue for 1,25-(OH)<sub>2</sub>-D<sub>3</sub> (Stumpf, W. E. et al., *Science*, 206:1188-1190 (1979)). Cells isolated from the skin of rats, mice, and humans, and from cultured human skin fibroblasts and keratinocytes contain a high affinity (1.0 × 10<sup>-10</sup> M) low capacity receptor-like protein for 1,25-dihydroxyvitamin D<sub>3</sub> (Franceschi, et al., *Arch. Biochem. Biophys.*, 210: 1-13 (1979); Simpson, R. U. et al., *P.N.A.S. (USA)*, 77: 5822 (1980); Colston, K. et al., *Endocrinology*, 107: 1916 (1980); Feldman, D. et al., *Journal of Clinical Endocrinology & Metabolism*, 51: 1463 (1980); Eil, C. et al., *P.N.A.S. (USA)*, 78: 2562 (1981); and Clemens, T. L. et al., *J. Clin. Endocr. Metab.* 56: April 1983). A specific biological function for 1,25-(OH)<sub>2</sub>-vitamin D<sub>3</sub> in the skin, however, has yet to be discovered. Nevertheless, evidence has come forth supporting the concept that the dihydroxy metabolite of the vitamin does have biologic actions in the skin. This evidence was obtained evaluating the biological activity of 1,25-dihydroxy vitamin D<sub>3</sub> simultaneously in cultured human skin fibroblasts that either possessed or lacked a cytosolic receptor-like protein for the hormone (Clemens, T. L. et al., *J. Clin. Endocrinol. Metab.*, 56: April 1983). The receptor-negative skin fibroblasts were obtained from a patient with a rare bone disorder called vitamin D dependent rickets, type II, a heritable disorder caused by a defective or complete absence of a cytoplasmic or nuclear receptor for 1,25-dihydroxyvitamin D. Administration of the dihydroxy metabolite of vitamin D<sub>3</sub> caused a dose-dependent inhibition of cell growth in receptor positive skin fibroblasts (about 40-50% reduction in cell growth was observed in cultures containing 10<sup>-6</sup> and 10<sup>-8</sup> M of hormone and 12% in cultures containing 10<sup>-10</sup> M of

1,25-(OH)<sub>2</sub>-D<sub>3</sub>), and, by contrast, had absolutely no effect on the growth of receptor negative skin fibroblasts.

Holick et al. (*New England Journal of Medicine*, 303:349-354 (1980)) have studied the feasibility of using the skin as an organ for the synthesis and absorption of vitamin D metabolites. These investigators demonstrated that topical application of various vitamin D metabolites or pro-vitamin forms followed by phototherapy results in elevated serum levels of dihydroxyvitamin D<sub>3</sub>. It was therefore suggested that topical application of vitamin D analogues may be an effective method of therapy for diseases involving calcium, phosphorous and bone metabolism problems.

Holick, U.S. Pat. No. 4,410,515, discloses vitamin D glycosides and their use in the regulation of calcium metabolism and phosphorous homeostasis.

Holick, U.S. Pat. No. 4,521,410, discloses water-soluble glycosyl orthoesters of vitamin D and their use in the regulation of calcium metabolism and phosphorous homeostasis.

Holick, U.S. Pat. No. 4,335,120, discloses that the toxic effects of orally administered vitamin D<sub>2</sub> and vitamin D<sub>3</sub> compounds can be avoided by topical administration whereby a slow and controlled transportation of the vitamin D compounds into the blood stream of a subject is achieved.

Jackson, U.S. Pat. No. 3,655,881 (1972) discloses methods for treating burned skin by inducing a state of calciphylaxis. Calciphylaxis is a hypersensitivity reaction resulting from the administration of or endogenous production of a sensitizing calcifier in combination with a challenger. Sensitizing calcifiers include, inter alia, vitamins D<sub>2</sub> and D<sub>3</sub>.

Dikstein, U.S. Pat. No. 4,610,478, and European Patent Application No. 0 129 003 (1984), discloses compositions containing 1-alpha-hydroxycholecalciferol or 1-alpha,25-dihydroxycholecalciferol for the topical treatment of skin disorders, such as dermatitis, psoriasis, eczema, solar keratosis and certain stages of wound healing and alopecia. Dikstein also teaches that low dosages are required, from about 0.03 µg to 1.0 µg per gram of composition, to minimize the risk of undesired side effects and systemic effects. However, Dikstein does not teach that vitamin D compounds are useful for the treatment of wounds caused by lacerations, punctures or cuts.

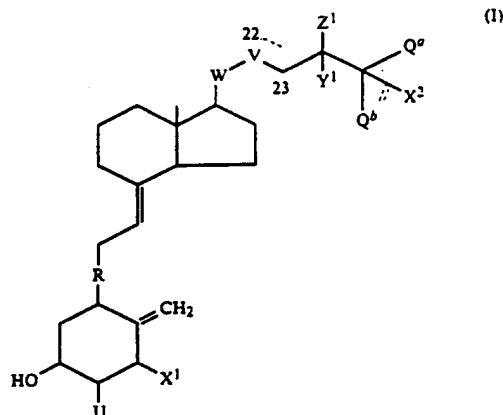
Additionally, no specific therapy is available for healing decubitus or diabetic ulcers of the feet, however it is suggested that a course of aggressive supportive treatment can lead to salvaging the limb. Therefore, a need exists for effective treatments for diabetic ulcers.

### SUMMARY OF THE INVENTION

Despite the teaching of Dikstein high doses of 1-alpha-hydroxycholecalciferol or 1-alpha,25-dihydroxycholecalciferol must be avoided, the inventors have discovered that the topical administration of relatively high levels of active vitamin D compounds and homologues, analogues, and hydroxylated metabolites thereof are therapeutically useful, in particular, for the treatment of wounds, ulcers, and periodontal disease.

In particular, the invention relates to a method of enhancing the healing of wounds in a patient comprises administering to said patient an effective amount of a vitamin D compound, wherein said vitamin D compound has the Formula (I):

4



wherein the bond between carbons C-22 and C-23 is single or double bond;

Y¹ is hydrogen, F, CH<sub>3</sub>, CH<sub>2</sub>CH<sub>3</sub> or X¹;

U is hydrogen, —OH or —O—(C<sub>2</sub>–C<sub>4</sub> alkyl)—OH;

Z¹ is F, H or X¹;

Qᵃ is CF<sub>3</sub> or CH<sub>2</sub>X¹;

Qᵇ is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

wherein X¹ is selected from the group consisting of hydrogen and —OH;

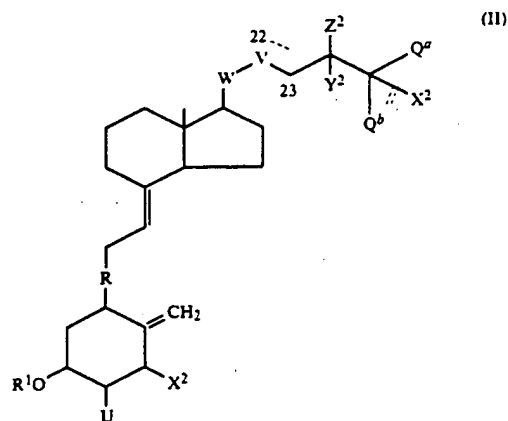
W is CH=CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O;

with the proviso that both W and V are not both O; and

"==" is either a single bond between Qᵃ and Qᵇ or a hydrogen atom on Qᵃ and Qᵇ, with the proviso that wherein "==" is a single bond, then X¹ is H.

The invention also relates to a method of enhancing the healing of wounds in a patient which comprises administering to said patient an effective amount of a vitamin D compound, wherein said vitamin D compound has the Formula (II):



wherein the bond between C-22 and C-23 is a single or double bond;

Y² is hydrogen, fluorine, methyl, ethyl or OR¹;

Z² is F, H or X²;

U is hydrogen, —OH or —O—(C<sub>2</sub>–C<sub>4</sub> alkyl)—OH;

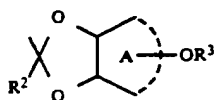
Qᵃ is CF<sub>3</sub> or CH<sub>2</sub>X²;

$Q^b$  is  $CF_3$  or  $CH_3$ ;

R is a double bond or an epoxy group;

$X^2$  is selected from the group consisting of hydrogen, and  $OR^1$ ;

$R^1$  is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or  $R^1$  is an orthoester glycoside moiety of the Formula (III):



wherein A represents a glucofuranosyl or glucopyranosyl ring;

$R^2$  is hydrogen, lower alkyl, aralkyl, or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower  $C_1$ - $C_4$  alkyl,  $C_1$ - $C_4$  alkoxy; or naphthyl; and

$R^3$  is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, with the proviso that at least one of the  $R^1$  is either a glycosidic residue or an orthoester glycoside moiety;

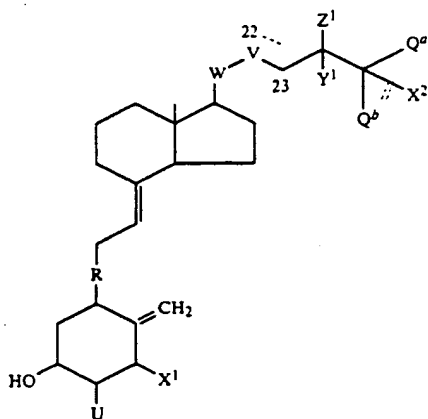
W is  $CH=CH_3$  or O; and

V is  $CH_2$  or O;

with the proviso that both W and V are not both O; and

"===" is either a single bond between  $Q^a$  and  $Q^b$  or a hydrogen atom on  $Q^a$  and  $Q^b$ , with the proviso that wherein "===" is a single bond, then  $X^1$  is H.

The invention also relates to a method of inhibiting scar formation in a patient arising from cuts, lacerations, puncture wounds and abrasions which comprises administering to said patient a pharmaceutical composition comprising an effective amount of a vitamin D compound and a pharmaceutically acceptable carrier, wherein said vitamin D compound has the Formula (I):



wherein the bond between carbons C-22 and C-23 is single or double bond;

$Y^1$  is hydrogen, F,  $CH_3$ ,  $CH_2CH_3$  or  $X^1$ ;

U is hydrogen,  $-OH$  or  $-O-(C_2-C_4 \text{ alkyl})-OH$ ;

$Z^1$  is F, H or  $X^1$ ;

$Q^a$  is  $CF_3$  or  $CH_2X^1$ ;

$Q^b$  is  $CF_3$  or  $CH_3$ ;

R is a double bond or an epoxy group;

wherein  $X^1$  is selected from the group consisting of hydrogen and  $-OH$ ;

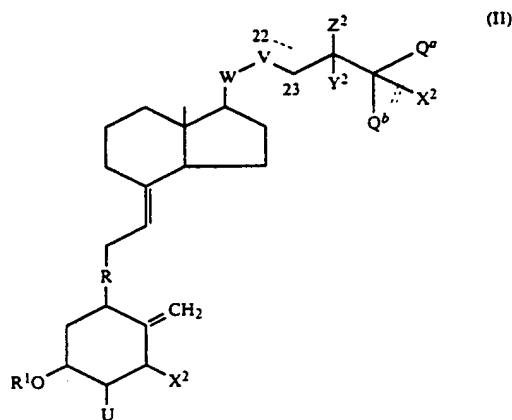
W is  $CH=CH_3$  or O; and

V is  $CH_2$  or O;

with the proviso that both W and V are not both O; and

"===" is either a single bond between  $Q^a$  and  $Q^b$  or a hydrogen atom on  $Q^a$  and  $Q^b$ , with the proviso that wherein "===" is a single bond, then  $X^1$  is H.

The invention also relates to a method for inhibiting scar formation in a patient arising from cuts, lacerations, puncture wounds and abrasions which comprises topically administering to said patient a pharmaceutical composition comprising an effective amount of a vitamin D compound and a pharmaceutically acceptable carrier, wherein said vitamin D compound has the Formula (II):



wherein the bond between C-22 and C-23 is a single or double bond;

$Y^2$  is hydrogen, fluorine, methyl, ethyl or  $OR^1$ ;

U is hydrogen,  $-OH$  or  $-O-(C_2-C_4 \text{ alkyl})-OH$ ;

$Z^2$  is F, H or  $X^2$ ;

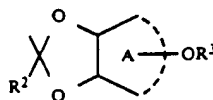
$Q^a$  is  $CF_3$  or  $CH_2X^2$ ;

$Q^b$  is  $CF_3$  or  $CH_3$ ;

R is a double bond or an epoxy group;

$X^2$  is selected from the group consisting of hydrogen, and  $OR^1$ ;

$R^1$  is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or  $R^1$  is an orthoester glycoside moiety of the Formula (III):



wherein A represents a glucofuranosyl or glucopyranosyl ring;

$R^2$  is hydrogen, lower alkyl, aralkyl, or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower  $C_1$ - $C_4$  alkyl,  $C_1$ - $C_4$  alkoxy; or naphthyl; and

$R^3$  is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue;

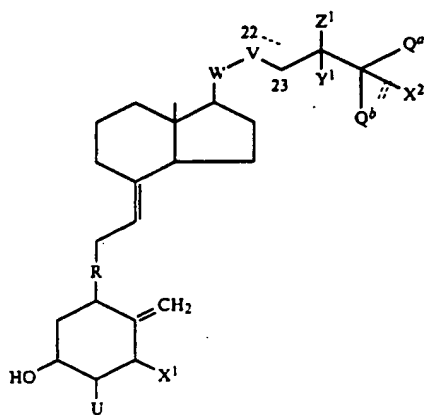
W is CH—CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O;

with the proviso that both W and V are not both O;  
and

"===" is either a single bond between Q<sup>a</sup> and Q<sup>b</sup> or  
a hydrogen atom on Q<sup>a</sup> and Q<sup>b</sup>, with the proviso  
that wherein "===" is a single bond, then X<sup>1</sup> is  
H.

The invention also relates to a method of treating  
gastric, duodenal, esophageal, decubitus, diabetic foot  
and genito-urinary ulcers in a patient which comprises  
administering to said patient a pharmaceutical composi-  
tion comprising an effective amount of a vitamin D  
compound and a pharmaceutically acceptable carrier,  
wherein said vitamin D compound has the Formula (I):



wherein the bond between carbons C-22 and C-23 is  
single or double bond;

Y<sup>1</sup> is hydrogen, F, CH<sub>3</sub>, CH<sub>2</sub>CH<sub>3</sub> or X<sup>1</sup>;

U is hydrogen, —OH or —O—(C<sub>2</sub>—C<sub>4</sub> alkyl)—OH;

Z<sup>1</sup> is F, H or X<sup>1</sup>;

Q<sup>a</sup> is CF<sub>3</sub> or CH<sub>2</sub>X<sup>1</sup>;

Q<sup>b</sup> is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

wherein X<sup>1</sup> is selected from the group consisting of  
hydrogen and —OH;

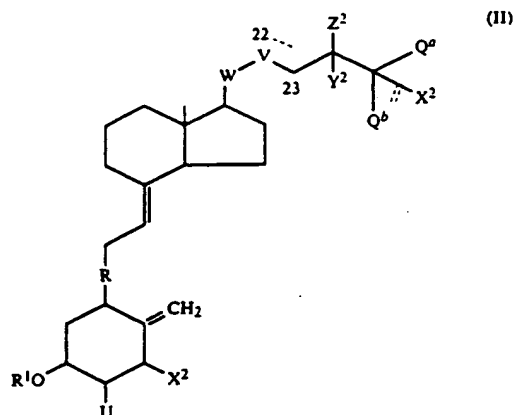
W is CH—CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O;

with the proviso that both W and V are not both O;  
and

"===" is either a single bond between Q<sup>a</sup> and Q<sup>b</sup> or  
a hydrogen atom on Q<sup>a</sup> and Q<sup>b</sup>, with the proviso  
that wherein "===" is a single bond, then X<sup>1</sup> is  
H.

The invention also relates to a method for treating  
gastric, duodenal, esophageal, decubitus, diabetic foot,  
genito-urinary ulcers and ulcerative keratitis in a patient  
which comprises topically or ophthalmically adminis-  
tering to said patient a pharmaceutical composition  
comprising an effective amount of a vitamin D com-  
pound and a pharmaceutically acceptable carrier,  
wherein said vitamin D compound has the Formula (II):



wherein the bond between C-22 and C-23 is a single  
or double bond;

Y<sup>2</sup> is hydrogen, fluorine, methyl, ethyl or OR<sup>1</sup>;

U is hydrogen, —OH or —O—(C<sub>2</sub>—C<sub>4</sub> alkyl)—OH;

Z<sup>2</sup> is F, H or X<sup>2</sup>;

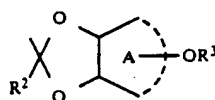
Q<sup>a</sup> is CF<sub>3</sub> or CH<sub>2</sub>X<sup>2</sup>;

Q<sup>b</sup> is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

X<sup>2</sup> is selected from the group consisting of hydrogen,  
and OR<sup>1</sup>;

R<sup>1</sup> is hydrogen or a straight or branched chain glyco-  
sidic residue containing 1–20 glycosidic units per  
residue, or R<sup>1</sup> is an orthoester glycoside moiety of  
the Formula (III):



wherein A represents a glucufuranosyl or  
glucopyranosyl ring;

R<sup>2</sup> is hydrogen, lower alkyl, aralkyl, or aryl, with the  
proviso that aryl is phenyl or phenyl substituted by  
chloro, fluoro, bromo, iodo, lower C<sub>1</sub>—C<sub>4</sub> alkyl,  
C<sub>1</sub>—C<sub>4</sub> alkoxy; or naphthyl; and

R<sup>1</sup> is hydrogen or a straight or branched chain glyco-  
sidic residue containing 1–20 glycosidic units per  
residue;

W is CH—CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O; with the proviso that both W and V  
are not both O; and

"===" is either a single bond between Q<sup>a</sup> and Q<sup>b</sup> or  
a hydrogen atom on Q<sup>a</sup> and Q<sup>b</sup>, with the proviso  
that wherein "===" is a single bond, then X<sup>1</sup> is  
H.

#### DESCRIPTION OF THE FIGURES

FIG. 1 depicts a graph showing the effect of vehicle  
and 5 μg, 10 μg, 27 μg and 54 μg of 1,25-dihydroxy-  
vitamin D<sub>3</sub> per gram of oil on the percentage of wound  
healing on days 1, 2, 3, 4, 5 and 6 in rats with experimen-  
tal wounds.

FIG. 2 depicts a graph showing the effect of vehicle  
and 27 μg 1,25-dihydroxyvitamin D<sub>3</sub>/gram oil on the  
percentage of wound healing on days 1, 2, 3, 4, 5 and 9  
in rats with experimental wounds.

# DESCRIPTION OF THE PREFERRED EMBODIMENTS

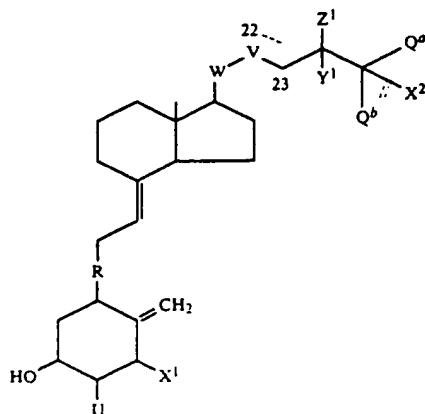
The present invention provides for a method of healing wounds and inhibiting scar formation. Wounds to the external epithelium include cuts, punctures and lacerations, including corneal lacerations. Wounds of the internal epithelium include peptic ulcers, esophageal mucosa injury, oral mucosa injuries and periodontal disease. The invention also provides for the treatment of ulcers such as diabetic ulcers of the feet, decubitus ulcers (bed sores), genito-urinary ulcers, peptic ulcers and ulcerative keratitis. Ulcerative keratitis is caused, for example, by extended wear of contact lenses.

As used herein, the term "septic ulcer" includes both duodenal and gastric ulcers. Peptic ulcers are a group of ulcerative disorders of the upper gastrointestinal tract; the primary forms of peptic ulcer are duodenal and gastric ulcer. Normally gastric mucosa has the ability to resist the corrosive effects of acid-pepsin. This is a trait which is unique to the gastric mucosa; areas such as the esophagus do not have this ability. Injury to the esophageal mucosa occurs from exposure to refluxed gastric juice and ulceration which occurs in the small intestine at the site of surgical attachment to actively secreting gastric mucosa.

Genito-urinary ulcers treatable with the vitamin D compounds of the invention include those caused by, for example, herpes simplex virus as well as other viral, fungal and bacterial infections. See Harrison's *Principles of Internal Medicine*, E. Braunwald et al. (eds.); McGraw-Hill Book Co., New York, N.Y., 1987, pp. 514-516.

The vitamin D compounds of the invention may also be administered for the treatment of periodontal disease. This disease begins as a marginal inflammation of the gingivae (gingivitis) which slowly spreads to involve the underlying alveolar bone and periodontal ligament. See Harrison's *Principles of Internal Medicine*, supra, pp. 164. The vitamin D compounds may be applied topically, for example, as part of a tooth paste formulation, or may be administered orally or part of a thin film implant between the teeth and gums to give sustained release of the vitamin D compound. Preferably, the film comprises a cellulose polymer. See U.S. Pat. No. 4,315,779 to Heyd.

Among the preferred compounds usable in the present invention are those of the formula (I):



wherein the bond between carbons C-22 and C-23 is single or double;

Y<sup>1</sup> is hydrogen, F, CH<sub>3</sub>, CH<sub>2</sub>CH<sub>3</sub> or X<sup>1</sup>;

U is hydrogen, —OH or —O—(C<sub>2</sub>–C<sub>4</sub> alkyl)—OH;

Z<sup>1</sup> is F, H or X<sup>1</sup>;

Q<sup>a</sup> is CF<sub>3</sub> or CH<sub>2</sub>X<sup>1</sup>;

Q<sup>b</sup> is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

X<sup>1</sup> and X<sup>2</sup> are selected from the group consisting of hydrogen and OH;

W is CH—CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O;

with the proviso that both W and V are not both O; and

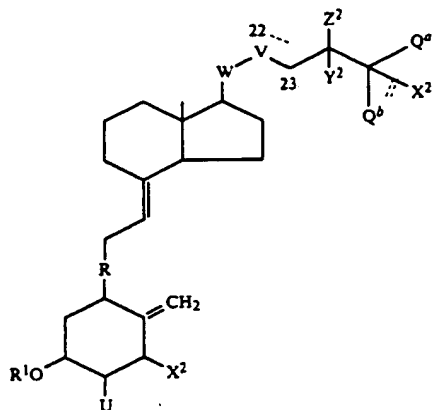
"= = =" is either a single bond between Q<sup>a</sup> and Q<sup>b</sup> or a hydrogen atom on Q<sup>a</sup> and Q<sup>b</sup>, with the proviso that wherein "= = =" is a single bond, then X<sup>1</sup> is H.

When the compounds of Formula (I) have a double bond at position C-22, they are derivatives of vitamin D<sub>2</sub>, whereas if the bond at that position is single, and there is a lack of C<sub>24</sub> alkyl, they are derivatives of vitamin D<sub>3</sub>. The latter are preferred.

Preferred are those compounds derived from vitamins D<sub>3</sub> or D<sub>2</sub>; 1-hydroxyvitamins D<sub>3</sub> or D<sub>2</sub>; 1,24-dihydroxyvitamins D<sub>2</sub> and D<sub>3</sub>; 1,25-dihydroxyvitamins D<sub>3</sub> and D<sub>2</sub>; 24,25-dihydroxyvitamins D<sub>3</sub> or D<sub>2</sub>; 25,26-dihydroxyvitamins D<sub>3</sub> or D<sub>2</sub>; 1,24,25-trihydroxyvitamins D<sub>3</sub> or D<sub>2</sub>. Most preferred among these are vitamins D<sub>3</sub> or D<sub>2</sub>; 1-hydroxyvitamins D<sub>3</sub> or D<sub>2</sub>; and 1,25-dihydroxyvitamins D<sub>3</sub> or D<sub>2</sub>; 5,6-epoxy derivatives of vitamin D and its metabolites, 2-β-(3-hydroxypropoxy)-1 α,25-dihydroxyvitamin D<sub>3</sub>, as well as the side chain fluoro derivatives of 1,25-(OH)<sub>2</sub> vitamin D and 1-(OH) vitamin D. Also preferred are 20- and 22-oxa vitamin D derivatives including 20-oxa-1α(OH)D<sub>3</sub>, 20-oxa-1α,25-(OH)<sub>2</sub>D<sub>3</sub>, 22-oxa-1α(OH)D<sub>3</sub> and 22-oxa-1α,25-(OH)<sub>2</sub>D<sub>3</sub> as well as pseudo-1-α-hydroxyvitamin D derivatives such as dihydrotachysterol and 5,6-trans vitamin D<sub>3</sub> and their 25-hydroxy derivatives.

Among other preferred compounds are water soluble derivatives of the aforementioned compounds of Formula (I) obtained by solubilizing such compounds by attaching thereto glycosidic residues such as those disclosed in Holick, U.S. Pat. No. 4,410,515. Alternative methods of solubilization are by conjugating compounds of Formula (I) to glycosyl orthoester residues, as disclosed in copending U.S. Ser. No. 607,117 by Holick et al., filed May 3, 1984. The disclosures of the aforementioned patent and application are herein incorporated by reference and made a part hereof.

Also useful in the practice of the invention are compounds of the Formula (II):



The vitamin D compounds are prepared or obtained according to the disclosures of the aforementioned references. In particular, the 5,6-epoxy derivatives of vitamin D<sub>3</sub> are obtained as described in *Jpn. Kokai Tokkyo Koho* JP 58,216,178 [83,216,178], Dec. 15, 1983. The fluoro derivatives are made or obtained as described in Shiina, et al., *Arch. Biochem. Biophys* 220:90 (1983). Methods for preparing the 20- and 22-oxa vitamin D derivatives are disclosed by Abe, J., et al., *Vitamin D Molecular, Cellular and Clinical Endocrinology* 310-319, Walter de Gruyter & Co., Berlin (1988). U.S. Pat. No.

The compounds can be employed in dosage forms such as tablets, capsules, powder packets, or liquid solutions, suspensions or elixirs for oral administration, sterile-liquid for formulations such as solutions or suspensions for parenteral use. Alternatively, the compounds can be present in a pharmacologically inert topical carrier such as one comprising a gel, an ointment or a cream, including such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters or mineral oils. Other possible carriers are liquid petroleum, isopropylpalmitate, polyethylene glycol ethanol 95%, polyoxyethylene monolaurate 5% in water, sodium lauryl sulfate 5% in water, and the like. Materials such as anti-oxidants, humectants, viscosity stabilizers and the like may be added, if necessary. The compounds may also be present as part of a cosmetic formulation which may be formulated according to methods known to those of skill in the art. The compounds can also be administered by means of pumps, tapes or patches.

Having now generally described this invention, the same will be understood by reference to the following examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

## EXAMPLES

Example 1 The Effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on Wound Healing in Rats

## Materials

25 CD rats 8 weeks old were obtained from Charles River Laboratories, Inc., Wilmington, Ma.

## Wounding Procedure

Rats were anesthetized with ether. Their backs were prepared by clipping and shaving. Two cutaneous wounds were made on each rat by punching on the right and left sides of the back with a sterile biopsy punch (diameter 4mm, wound thickness: full thickness of skin).

Control rats received vehicle only on wounds (20  $\mu$ l vegetable oil/two wounds/day for 4 days). The other four groups of rats (5 rats per group) were used to study the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on wound healing at different doses (5  $\mu$ g 1,25(OH)<sub>2</sub>D<sub>3</sub>/g Oil, 10  $\mu$ g/g oil, 27  $\mu$ g/g oil and 54  $\mu$ g/g oil 1,25(OH)<sub>2</sub>D<sub>3</sub>). Treatment continued for up to 4 days.

## Measurement Procedure

Wound area was estimated by planimetry. For this purpose, the wound was covered with a transparent plastic film and wound outlines were drawn with a marker. Wound shape was then magnified, was cut out, and weighed.

Healing was assessed as the decrease in wound area on days 2, 3, 4, 5 and 6. The results are shown in Table I below and is depicted in FIG. 1. The data (percent healing on day 2, 3, 4, 5 and 6) were analyzed for significance using students T test.

In order to confirm the effectiveness of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in promoting wound healing, a second experiment was carried out using eight rats (dose of 1,25(OH)<sub>2</sub>D<sub>3</sub>=27  $\mu$ g/g oil). The results are depicted in FIG. 2.

## Results

As can be seen clearly in FIGS. 1 and 2, topical administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> enhanced substantially the healing of puncture wounds in rats. The extent of wound healing was directly related to the concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in oil applied to the wound. These results demonstrate conclusively that vitamin D compounds are useful for enhancing wound healing in individuals.

TABLE 1

GROUP	% HEALING <sup>a</sup>				
	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
1. CONTROL	-8 $\pm$ 5 <sup>b</sup>	14 $\pm$ 3	19 $\pm$ 4	41 $\pm$ 6	60 $\pm$ 5
2. 1,25 (OH) <sub>2</sub> D <sub>3</sub> (5 $\mu$ g/g oil)	1 $\pm$ 5	14 $\pm$ 7	23 $\pm$ 6	52 $\pm$ 5	67 $\pm$ 5
3. 1,25 (OH) <sub>2</sub> D <sub>3</sub> (10 $\mu$ g/g oil)	2 $\pm$ 4	19 $\pm$ 4	32 $\pm$ 3 <sup>§</sup>	50 $\pm$ 3	64 $\pm$ 3
4. 1,25 (OH) <sub>2</sub> D <sub>3</sub> (27 $\mu$ g/g oil)	7 $\pm$ 4 <sup>§</sup>	21 $\pm$ 3	34 $\pm$ 5 <sup>§</sup>	48 $\pm$ 4	64 $\pm$ 4
5. 1,25 (OH) <sub>2</sub> D <sub>3</sub>	7 $\pm$ 4 <sup>§</sup>	21 $\pm$ 4	37 $\pm$ 4 <sup>#</sup>	57 $\pm$ 4 <sup>§</sup>	72 $\pm$ 2*

TABLE 1-continued

EFFECT OF 1,25(OH) <sub>2</sub> D <sub>3</sub> ON WOUND HEALING OF RATS					
GROUP	% HEALING <sup>a</sup>				
	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6

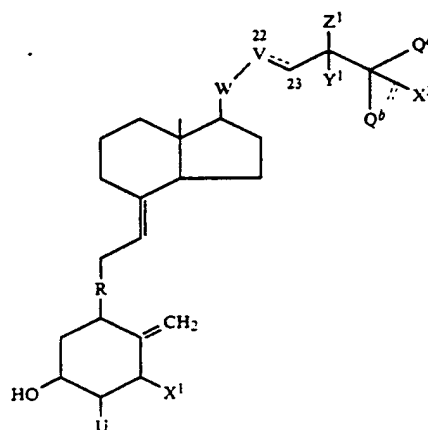
$$\% \text{ HEALING} = \frac{\text{Original Wound Area} - \text{Lesion Area}}{\text{Original Wound Area}} \times 100\%$$

<sup>a</sup>MEANS  $\pm$  SEM, n=10

10 Significance of difference from control using Student's t test: \* p < .05, § p < .025 and # p < .005

What is claimed as new and desired to be covered by U.S. Letters Patent is:

1. A method of treating periodontal disease in an animal which comprises administering to said animal a pharmaceutical composition comprising a therapeutically effective amount of a vitamin D compound and a pharmaceutically acceptable carrier, wherein said vitamin D compound has the formula:



wherein the bond between carbons C-22 and C-23 is single or double bond;

Y<sup>1</sup> is hydrogen, F, CH<sub>3</sub>, CH<sub>2</sub>CH<sub>3</sub> or X<sup>1</sup>;

U is hydrogen, —OH or —O—(C<sub>2</sub>–C<sub>4</sub> alkyl)—OH;

Z<sup>1</sup> is F, H or X<sup>1</sup>;

Q<sup>a</sup> is CF<sub>3</sub> or CH<sub>2</sub>X<sup>1</sup>;

Q<sup>b</sup> is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

wherein X<sup>1</sup> and X<sup>2</sup> are selected from the group consisting of hydrogen and —OH;

W is CH—CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O; with the proviso that both W and V are not both O; and

"=" is either a single bond between Q<sup>a</sup> and Q<sup>b</sup> or a hydrogen atom on Q<sup>1</sup> and Q<sup>b</sup>, with the proviso that wherein "=" is a single bond, then X<sup>2</sup> is H.

2. The method of claim 1, wherein said bond between carbons C-22 and C-23 is a single bond and X<sup>1</sup> is hydrogen.

3. The method of claim 1, wherein said bond between C-22 and C-23 is a single bond, X<sup>1</sup> is hydroxyl, and at least one of the group consisting of Y<sup>1</sup>, Z<sup>1</sup>, Q<sup>a</sup> and Q<sup>b</sup> contains a fluorine atom.

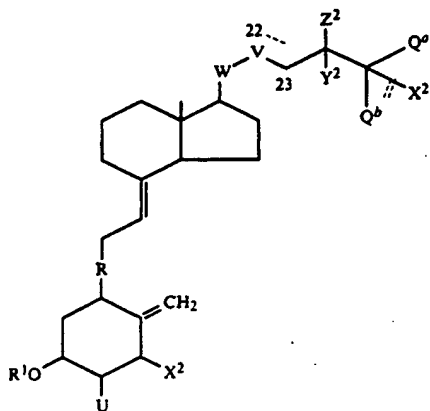
4. The method of claim 1, wherein said bond between C-22 and C-23 is a double bond.

5. The method of claim 1, wherein said bond between C-22 and C-23 is a double bond and X<sup>1</sup> is hydrogen.

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6. The method of claim 1, wherein said bond between C-22 and C-23 is a double bond and X<sup>1</sup> is hydroxyl.

7. A method of treating periodontal disease in an animal which comprises administering to said animal a pharmaceutical composition comprising an effective amount of a vitamin D compound and a pharmaceutically acceptable carrier, wherein said vitamin D compound has the formula:



wherein the bond between carbons C-22 and C-23 is single or double bond;

Y<sup>2</sup> is hydrogen, fluorine, methyl, ethyl or OR<sup>1</sup>;

U is hydrogen, —OH or —O—(C<sub>2</sub>–C<sub>4</sub> alkyl)—OH;

Z<sup>2</sup> is F, H or X<sup>2</sup>;

Q<sup>a</sup> is CF<sub>3</sub> or CH<sub>2</sub>X<sup>2</sup>;

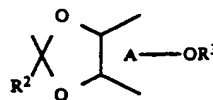
Q<sup>b</sup> is CF<sub>3</sub> or CH<sub>3</sub>;

R is a double bond or an epoxy group;

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X<sup>2</sup> is selected from the group consisting of hydrogen and —OR<sup>1</sup>;

wherein R<sup>1</sup> is hydrogen or a straight or branched chain glycosidic residue containing 1–20 glycosidic units per residue, or R<sup>1</sup> is an orthoester glycoside moiety of the formula:



wherein A represents a glucofuranosyl or glucopyranosyl ring;

R<sup>2</sup> is hydrogen, lower alkyl, aralkyl, or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower C<sub>1</sub>–C<sub>4</sub> alkyl, C<sub>1</sub>–C<sub>4</sub>; or naphthyl; and

R<sup>3</sup> is hydrogen or a straight or branched chain glycosidic residue containing 1–20 glycosidic units per residue, with the proviso that said vitamin D compound has at least one R<sup>1</sup> which is a glycosidic residue or an orthoester glycoside moiety;

W is CH—CH<sub>3</sub> or O; and

V is CH<sub>2</sub> or O; with the proviso that both W and V are not both O; and

"=" is either a single bond between Q<sup>1</sup> and Q<sup>b</sup> or a hydrogen atom on Q<sup>a</sup> and Q<sup>b</sup>, with the proviso that wherein "=" is a single bond, then X<sup>2</sup> at C-25 is H.

8. The method of claim 7, wherein said bond between C-22 and C-23 is a single bond and at least one of the group consisting of Y<sup>1</sup>, Z<sup>1</sup>, Q<sup>a</sup> and Q<sup>b</sup> contains a fluorine atom.

\* \* \* \* \*



US005700790A

**United States Patent** [19]

Gulbrandsen et al.

[11] Patent Number: **5,700,790**[45] Date of Patent: **\*Dec. 23, 1997****[54] PREVENTION AND TREATMENT OF MYOCARDIAL FAILURE**

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[\*] Notice: The term of this patent shall not extend  
beyond the expiration date of Pat. No.  
5,350,745.

[21] Appl. No.: **588,067**

[22] Filed: **Jan. 17, 1996**

**Related U.S. Application Data**

[63] Continuation of Ser. No. 311,934, Sep. 26, 1994, abandoned,  
which is a continuation of Ser. No. 10,823, Jan. 29, 1993,  
Pat. No. 5,350,745.

[51] Int. Cl.<sup>6</sup> ..... **A61K 31/59**

[52] U.S. Cl. .... **514/167**

[58] Field of Search ..... **514/167, 168**

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**Primary Examiner—William R. A. Jarvis**

**Attorney, Agent, or Firm—Teresa J. Welch; Stroud, Stroud,**  
**Willink, Thompson & Howard**

**[57] ABSTRACT**

Method of increasing the strength of contraction in the  
mammalian heart muscle by administering to the mammal  
an effective amount of an activated Vitamin D compound,  
i.e. a 1 $\alpha$ -hydroxylated Vitamin D compound which binds  
with the Vitamin D receptor and produces a positive ino-  
tropic effect in the heart muscle. The activated Vitamin D  
compound may be given as a means to prevent myocardial  
failure or to treat myocardial failure.

**7 Claims, No Drawings**

# PREVENTION AND TREATMENT OF MYOCARDIAL FAILURE

This application is a continuation of application Ser. No. 08/311,934 filed on Sep. 26, 1994, now abandoned, which is a continuation of application Ser. No. 08/010,823 filed Jan. 29, 1993, now U.S. Pat. No. 5,350,745

## FIELD OF THE INVENTION

This application claims priority to PCT/US94/01172 filed Jan. 31, 1994.

This invention relates a method of treating myocardial failure, more specifically it relates to the use of active forms of vitamin D to increase the strength contraction of the heart muscle.

## BACKGROUND OF THE INVENTION

Heart failure is a common clinical condition and results in a significant morbidity and mortality. It is defined as the pathophysiologic state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues or can do so only from an abnormally elevated filling pressure. Heart failure is frequently, but not always caused by a defect in myocardial contraction wherein the strength of contraction of the heart muscle is diminished. In such a case, the term myocardial failure is appropriate. Few therapies exist for myocardial failure that are effective and do not present significant undesirable side effects. The most common treatment of myocardial failure is the administration of cardiac glycosides such as digitalis. While digitalis can alleviate the symptoms and improve cardiac hemodynamics in heart failure, it, as well as the other cardiac glycosides, has a low margin of safety. Such potent drugs cause cardiac dysrhythmias and neurological problems as well as nausea, abdominal pain and headache. Further, drug interaction problems are reported with the cardiac glycosides and other common drugs.

What is needed is a method of increasing the strength of the heart contraction without the above described undesirable side effects.

## DESCRIPTION OF THE INVENTION

The present invention is for a method of treating myocardial failure using an active form of vitamin D. Vitamin D is known to be important in the regulation of calcium metabolism in animals and man. See, *Harrison's Principles of Internal Medicine: Part Eleven, "Disorders of Bone and Mineral Metabolism"*, Chapter 335, E. Braunwald, et al., (eds.), McGraw-Hill, New York, 1987, pp. 1860-1865.

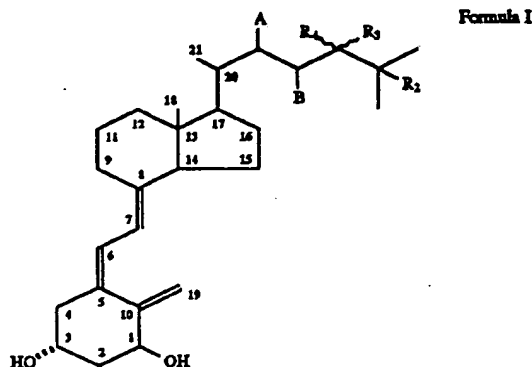
It is known that vitamin D<sub>3</sub> must be hydroxylated in the 1 and the 25 position before it is activated i.e. before it will produce a biological response. A similar metabolism appears to be required to activate the other forms of vitamin D e.g. vitamin D<sub>2</sub> and vitamin D<sub>4</sub>. As is generally understood and used herein, the term "vitamin D" is intended to include vitamins D<sub>3</sub>, D<sub>2</sub>, and D<sub>4</sub>. The term activated vitamin D, as used herein, is intended to refer to vitamin D which has been hydroxylated in at least the 1 position of the A ring and binds with the vitamin D receptor. e.g. 1,25-dihydroxyvitamin D<sub>3</sub>.

The 1 $\alpha$ -hydroxyvitamin D of the present invention has the general formula described in formula I wherein A and B are either hydrogen or a carbon to carbon bond thus forming a double bond between C22 and C23, R<sub>1</sub> and R<sub>2</sub> can be either hydrogen, hydroxy, lower alkyl, O-lower alkyl, O-lower

acyl, O-aromatic acyl or fluoro, and where R<sub>3</sub> is hydrogen or lower alkyl along with an acceptable excipient.

In the formulae shown in this specification and in the claims a wavy line to substituent X indicates that the substituent can be either  $\alpha$  or  $\beta$  stereoisomeric form. Wherever in this specification and in the claims the word "lower" is used as a modifier of alkyl or acyl it is intended to identify a hydrocarbon chain having from about 1 to 4 carbon atoms and can be either a straight chain or branched chain configuration.

Specific examples of such hydrocarbon chains are: methyl, ethyl, propyl, butyl, isobutyl or t-butyl, and formyl, acetyl, propionyl, or butyryl. The word "aromatic acyl" as used herein and in the claims is meant to identify a benzoyl group or a substituted benzoyl group such as nitrobenzoyl or dinitrobenzoyl.



Among the preferred active vitamin Formula I D compounds are:

- 1 $\alpha$ ,25-dihydroxy-cholecalciferol [1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>3</sub>]
- 1 $\alpha$ -hydroxy-cholecalciferol [1 $\alpha$ -(OH)D<sub>3</sub>]
- 1 $\alpha$ ,24-dihydroxy-cholecalciferol [1 $\alpha$ ,24-(OH)<sub>2</sub>D<sub>3</sub>]
- 1 $\alpha$ ,25-dihydroxy-ergocalciferol [1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>2</sub>]
- 1 $\alpha$ -hydroxy-ergocalciferol [1 $\alpha$ -(OH)D<sub>2</sub>]
- 1 $\alpha$ ,24(s)-dihydroxy-ergocalciferol [1 $\alpha$ ,24(s)-(OH)<sub>2</sub>D<sub>2</sub>]
- 1 $\alpha$ ,25-dihydroxy-vitamin D<sub>4</sub> [1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>4</sub>]
- 1 $\alpha$ -hydroxy-vitamin D<sub>4</sub> [1 $\alpha$ -(OH)D<sub>4</sub>]
- 1 $\alpha$ ,24-dihydroxy-vitamin D<sub>4</sub> [1 $\alpha$ ,24-(OH)<sub>2</sub>D<sub>4</sub>]

The above described active forms of vitamin D can be prepared as described in U.S. Pat. Nos. 3,993,675; 4,022,891; 4,195,027; 4,234,495; 4,508,651 and co-pending U.S. applications 07/940,246 and 07/991,493 all incorporated herein by reference.

Functionally, vitamin D is more appropriately considered a hormone than a vitamin. When activated, vitamin D interacts with a vitamin D receptor protein and this interaction ultimately results in some form of biological response. For example, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> is known to be a potent stimulator of calcium absorption from the intestine which is mediated by the interaction of the 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> molecule and the vitamin D receptor protein located in the epithelial cells (enterocytes) which line the intestine.

In recent years it has become evident that the vitamin D receptor protein is widely distributed in the bodies of animals and man. Thus, it is not surprising that besides calcium homeostasis, activated vitamin D has been implicated in osteogenesis, modulation of immune response, modulation of the process of insulin secretion by the pancreatic B cell, muscle cell function and the differentiation and growth of epidermal and hemopoietic tissues.

More recently, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> receptors have been shown to exist in the rat heart (Walters et al., J.

Mol. Cell Cardiol. 18:67-72 (1986)) and this has prompted the speculation that vitamin D may play a role in cardiac function. Until the present invention, the prevailing view, which was based on studies of cardiac hemodynamics in vitamin D<sub>3</sub> deficient rats, was that 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> produced a direct negative inotropic effect in the heart, presumably by promoting the sequestering of calcium in the myocardium. (Weisharr and Simpson, Am. J. Physiol. 253 (Endocrinol. Metab. 16): E675-E683 (1987).

Contrary to the hypothesis of Weisharr and Simpson, the present inventors have found that active forms of vitamin D, including 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> produce a direct positive inotropic effect in the mammalian myocardium i.e. increases the strength of the contraction of the heart muscle.

#### Example 1: Positive inotropic effect

Rat right ventricular papillary muscles were mounted in an experimental chamber and stimulated at 0.3-0.7 Hz with a single pulse, broad field stimulation via platinum plate electrodes. The preparation was continuously perfused at 22°-24° with oxygenated modified Tyrode's solution, pH 7, containing 2 mM Ca<sup>2+</sup>. Twitch tension of the preparation was measured by suturing one end of the preparation to a force transducer and the other end to a three way positioner. Muscles attaining a stable baseline twitch tension were then perfused with 0.1 to 6.25  $\mu$ M of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. In nine experiments 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> increased steady-state twitch tension an average 14 $\pm$ 11% (range of 4-41%). The effects of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> were reversed by drug washout. These results indicate that 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> has a positive inotropic effect on the mammalian myocardium.

#### Example 2: prevention of Congestive Heart Failure

An oral dosage formulation containing 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> is evaluated in a double blind study for efficacy in the preventing the development of heart failure caused by myocardial failure. The formulation evaluated contains 0.25  $\mu$ g of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. The control formulation is identical except that it does not contain the 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Five hundred normal subjects between the ages of 55 and 65 are selected. The subjects are divided into an experimental and control population. They are instructed to take the medication twice a day, in the morning and in the evening.

Evaluations of cardiovascular hemodynamics, are conducted at six month intervals by a physician. The final evaluation is carried out at the end of three years of preventive therapy. The results of the study show that daily oral administration of 1,25-dihydroxyvitamin D<sub>3</sub> significantly reduces the occurrence of myocardial failure in the experimental group as compared with the control.

As the above example illustrates, preventive benefit in reducing the occurrence of myocardial failure is derived from daily administration of a relatively low dosage of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. For treatment purposes, however, a higher dosage would be desired. However, the vitamin D<sub>3</sub> compounds, particularly, 1 $\alpha$ ,25-

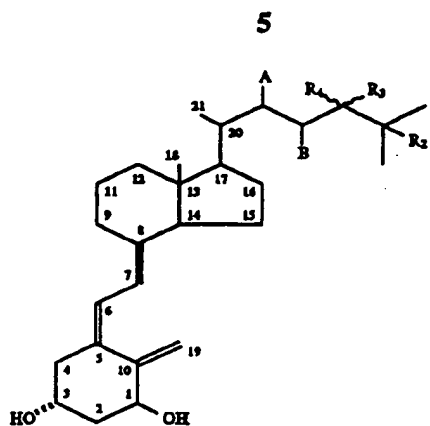
dihydroxyvitamin D<sub>3</sub> cannot safely be administered at a dosage greater than 1.0  $\mu$ g per day without causing hypercalcemia and hypercalciuria in a large portion of the population. In that regard the active forms of vitamin D<sub>2</sub> and vitamin D<sub>4</sub> are more suitable for while they display a high binding activity with respect to the vitamin D receptor they have a much lower calcemic effect and are thus much less toxic. See for example co-pending U.S. application Ser. No. 07/940,246 which is incorporated herein by reference. Preferred in this regard are 1 $\alpha$ -hydroxy-ergocalciferol[1 $\alpha$ -(OH)D<sub>2</sub>], 1 $\alpha$ ,24(s)-dihydroxy-ergocalciferol[1 $\alpha$ ,24(s)-(OH)<sub>2</sub>D<sub>2</sub>], 1 $\alpha$ -hydroxy-vitamin D<sub>4</sub>[1 $\alpha$ -(OH)D<sub>4</sub>] and 1 $\alpha$ ,24-dihydroxy-vitamin D<sub>4</sub>[1 $\alpha$ ,24-(OH)<sub>2</sub>D<sub>4</sub>].

Advantageously, the vitamin D<sub>2</sub> and D<sub>4</sub> compounds of the present invention or combinations thereof with other therapeutic agents can be administered in dosage amounts of from 0.1 to 10.0 micrograms per day. These compounds can be administered as sterile parenteral solutions by injection or intravenously or by alimentary canal in the form of oral dosages, or by suppository. In relation to treatment of early stage myocardial failure doses from about 1.5 to about 6.0 micrograms per day are generally effective. For more advanced stages of myocardial failure, it may be advisable to administer the compounds of the present invention in conjunction with more traditional therapies such as the cardiac glycosides. Surprisingly it is found that the compounds of the present invention produce a synergistic response when administered in conjunction with another positive inotropic compound such as the glycosides. This synergistic effect allows the physician to administer a lower dosage of the glycosides and helps to avoid many of the undesirable side effects of the glycosides. If the compounds of the present invention are administered in combination with other therapeutic agents, the proportions of each of the compounds in the combination being administered will be dependent on the particular agents being used and the degree of heart failure being treated. It being understood that the specific dosage administered in any given case will be adjusted in accordance with the specific compounds being administered, the stage of the myocardial failure to be treated, the condition of the subject and the other relevant medical facts that may modify the activity of the drug or the response of the subject, as is well known by those skilled in the art.

While the present invention has now been described and exemplified with some specificity, those skilled in the art will appreciate the various modifications, including variations, additions, and omissions, that may be made in what has been described. Accordingly, it is intended that these modifications also be encompassed by the present invention and that the scope of the present invention be limited solely by the broadest interpretation that lawfully can be accorded the appended claims.

What is claimed is:

1. A method for preventing myocardial failure in a mammal in need thereof comprising administering to said mammal an effective amount of a compound of the general structure of Formula I



Formula I

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wherein A and B are either hydrogen or a carbon to carbon bond thus forming a double bond between C22 and C23,  $R_1$  and  $R_2$  can be either hydrogen, hydroxy, lower alkyl, O-lower alkyl, O-lower acyl, O-aromatic acyl or fluoro, and where  $R_3$  is hydrogen or lower alkyl along with an acceptable excipient.

2. A method for preventing myocardial failure as described in claim 1 wherein the compound of Formula I is selected from the group consisting of  $1\alpha,25$ -dihydroxycholecalciferol,  $1\alpha$ -hydroxy-cholecalciferol,  $1\alpha,24$ -dihydroxy-cholecalciferol,  $1\alpha,25$ -dihydroxy-ergocalciferol,  $1\alpha$ -hydroxy-ergocalciferol,  $1\alpha,24(s)$ -dihydroxy-ergocalciferol,  $1\alpha,25$ -dihydroxy-vitamin  $D_3$ ,  $1\alpha$ -hydroxy-vitamin  $D_3$ ,  $1\alpha,24$ -dihydroxy-vitamin  $D_3$ .

3. A method for reducing the occurrence of myocardial failure in a mammal in need thereof comprising administering to the mammal an effective amount of a compound of general Formula I

wherein A and B are either hydrogen or a carbon-to-carbon bond, thus forming a double bond between C-22 and C-23,  $R_1$  and  $R_2$  are either hydrogen, hydroxy, lower alkyl, O-lower alkyl, O-lower acyl, O-aromatic acyl or fluoro, and  $R_3$  is hydrogen or lower alkyl along with an acceptable excipient.

4. The method of claim 3 wherein said amount is 0.25  $\mu\text{g}$  administered twice daily.

5. The method of claim 3, wherein A and B are hydrogen and  $R_3$  is methyl or A and B form a double bond and  $R_3$  is methyl.

6. The method of claim 5 wherein said amount is from 0.1  $\mu\text{g}$  to 10.0  $\mu\text{g}$  per day.

7. The method of claim 3 wherein said compound is administered in combination with a cardiac glycoside.

\* \* \* \* \*

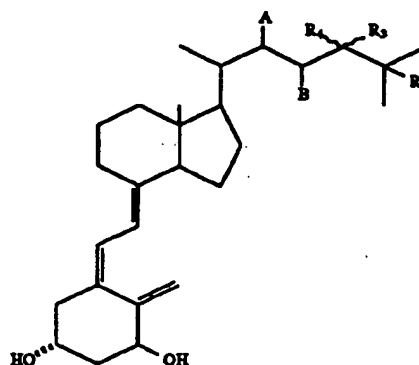
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Formula I



APPENDIX III

EXCERPT FROM FILE WRAPPER OF KNUTSEN ET AL.  
U.S. PATENT NO. 5,488,120



**CERTIFICATE OF EXPRESS MAIL**

I hereby certify that this paper (along with any paper referred to as being attached enclosed) is being deposited with the United States Postal Service in an envelope as "Express Mail Post Office to Addressee", Mailing Label No. TB550499917US addressed to: Commissioner of Patents and Trademarks, Box FWC, Washington, DC 20231,

On: August 24, 1994

By: Phyllis M. Leach

**IN THE UNITED STATES PATENT & TRADEMARK OFFICE**

**Applicant:** Knutson, et al.

**Docket No.:** 7982.79

**Serial No.:**

**Art Unit:** 1206

**Filed:** August 24, 1994

**Examiner:** Kestler, K.

**For:** NOVEL  $1\alpha$ -HYDROXY VITAMIN D<sub>4</sub> AND NOVEL INTERMEDIATES AND ANALOGUES

**DECLARATION UNDER 37 CFR 1.132**

Hon. Commissioner of Patents  
and Trademarks  
Washington, D.C. 20231

Sir:

I, Joyce C. Knutson, hereby declare and state the following:

1. I am Director of Preclinical Research at Lunar Corporation, the assignee of the above-identified patent application.

2. I received a Bachelor of Arts in Chemistry from Carleton College in 1968 and the degree of Doctor of Philosophy in Biochemistry from the University of Wisconsin - Madison. Since 1968 to the present I

have worked extensively and conducted research on Vitamin D compounds, their chemistry and metabolism. I consider myself an expert in the biochemistry and metabolism of vitamin D. Attached hereto as Exhibit A and incorporated by reference is a copy of my curriculum vitae.

3. I am one of the joint inventors of claims 3 and 5 of the present patent application.

4. I have reviewed the Official Action dated 24 September 1993. I am offering this declaration to correct alleged insufficiencies which the Examiner identified in my prior declarations.

5. Under my direction, LUNAR Corporation ("LUNAR") has completed a comparison of the biological activity of  $1\alpha$ -hydroxy Vitamin D<sub>4</sub>,  $1\alpha$ -hydroxy Vitamin D<sub>3</sub> (the compound of the cited references which the examiner has opined is the closest related compound to  $1\alpha$ -hydroxy Vitamin D<sub>4</sub>), and 1,25 dihydroxy Vitamin D<sub>3</sub> (the compound considered the prototype against which other vitamin D compounds are measured). The method employed in the comparative study is as follows:

a. The study was conducted using vitamin D deficient weanling rats. Initially, two large groups (201 on one occasion and 212 on another) were fed a vitamin D deficient diet (0.47% calcium, 0.3% phosphorus) for three weeks. Animals then were randomly selected from these vitamin deficient animals and randomly placed into groups of 8 to 12 rats each for conducting the studies. One control group of animals from each vitamin deficient group was selected, i.e., there were two control groups.

b. The experimental groups were administered doses of test compound at 0.042, 0.250 and 1.500 mcg/kg/day, with the control group rats receiving a comparable quantity of the vehicle, Fractionated Coconut Oil. All doses were administered by gavage once daily for fourteen days.

c. Animals were observed for clinical signs and mortality once daily. Body weights and food consumption were recorded weekly. Body weights of the rats varied at the initiation of the study and ranged from 123 to 217 grams. Statistically significant increases in mean body weight were observed on Days 7 and 14 in all three dose levels of all three test groups when compared with control. Mean food

consumption in all three dose levels of all three test articles revealed a statistically significant increase when compared with control. Two rats died in one of the control groups. The mortality that occurred was not dose related, there was no obvious cause of the deaths on autopsy and the belief is that the deaths are related to the high incidence of mortality observed in this animal model prior to test administration. No mortality was observed in any of the animal groups receiving test compounds. At termination of the study all surviving animals were fasted overnight and blood was withdrawn. Serum calcium determinations were completed. The raw data for the calcium determination for each animal in each group are assembled in Exhibit B incorporated herein by reference.

6. Table 1 shows the results of the above-described study. These results indicate that  $1\alpha$ -hydroxy Vitamin D<sub>4</sub> is essentially equivalent to  $1\alpha$ -hydroxy Vitamin D<sub>3</sub> and 1,25 dihydroxy Vitamin D<sub>3</sub> in its ability to stimulate an increase in serum calcium. This experimental comparison confirms the comparison with the literature reported in my declaration dated November 17, 1992 which had previously been filed in the parent case to the present, above-referenced application.

**Table 1**

<b><math>1\alpha</math>-(OH) Vitamin D<sub>4</sub></b>		<b><math>1\alpha</math>-(OH) Vitamin D<sub>3</sub></b>		<b>1,25 (OH)<sub>2</sub> Vitamin D<sub>3</sub></b>	
Dosage (mcg/kg/day)	Serum Calcium Concentration (mg/100ml) ± Standard Deviation	Dosage (mcg/kg/day)	Serum Calcium Concentration (mg/100ml) ± Standard Deviation	Dosage (mcg/kg/day)	Serum Calcium Concentration (mg/100ml) ± Standard Deviation
0.042	7.2 ± 1.19	0.042	9.0 ± 1.31	0.042	8.0 ± 1.51
0.250	12.1 ± 1.04	0.250	12.0 ± 0.90	0.250	8.5 ± 1.21
1.500	12.1 ± 0.69	1.500	12.9 ± 0.97	1.500	12.0 ± 0.60

7. Under my direction, LUNAR determined the median lethal dose ( $LD_{50}$ ) of  $1\alpha$ -hydroxy Vitamin  $D_4$  and compared that to the  $LD_{50}$  for  $1\alpha$ -hydroxy Vitamin  $D_2$ . The determinations were done in young adult (8 to 10 weeks) male and female rats. The male rats ranged in weight from 215 to 296 grams. The female rats were 160 to 180 grams in weight. The test compound was administered as a single oral dose at dosage levels of 0.32, 0.63, 1.25 and 2.5 mg/kg to male rats, and 1.25, 2.5 and 5.0 mg/kg to female rats. The test compound was prepared in Fractionated Coconut Oil and administered at a dose volume of 2.0 ml/kg. Five male and/ or five females comprised each dosage level. The duration of the study was 15 days.

8. The  $LD_{50}$  value for  $1\alpha$ -hydroxy Vitamin  $D_4$  (with 95% confidence limits) for combined male and female rats was calculated to be 1.67 (1.16 - 2.42) and the  $LD_{50}$  value for  $1\alpha$ -hydroxy Vitamin  $D_2$  (with 95% confidence limits) for combined male and female rats was calculated to be 1.8 (1.4 - 2.3) mg/kg. The data for these results are provided in Exhibit C, which is incorporated herein by reference. These results indicate that the toxicity of  $1\alpha$ -hydroxy Vitamin  $D_4$  is equivalent to that of  $1\alpha$ -hydroxy Vitamin  $D_2$  which has been shown by Sjoden et al., Proc. Soc. 178, 432-436 (1985) to be 5 to 15 times less toxic than  $1\alpha$ -hydroxy Vitamin  $D_3$ .

9. Under my direction, LUNAR examined the binding of 1,24-dihydroxy vitamin  $D_4$  to the vitamin D receptor. We found in these experiments that 1,24-dihydroxy vitamin  $D_4$  bound 1-2 times less tightly to the vitamin D receptor than does 1,25-dihydroxy vitamin  $D_3$ . This indicates that 1,24-dihydroxy vitamin  $D_4$  has high affinity for the vitamin D receptor. These data are consistent with gene expression studies done by LUNAR with 1,24-dihydroxy vitamin  $D_4$  which demonstrated that 1,24-dihydroxy vitamin  $D_4$  was 1-2 fold less active than was 1,25-dihydroxy vitamin  $D_3$ . These data indicate that 1,24-dihydroxy vitamin  $D_4$  has significant biological activity. A description of these experiments and the data are provided in

Exhibit D, hereto, incorporated herein by reference.



10. Under my direction, LUNAR examined the binding of 1,24-dihydroxy vitamin D<sub>4</sub> to the serum vitamin D binding protein. The experiments demonstrated that 1,24-dihydroxy vitamin D<sub>4</sub> bound the serum vitamin D protein ten-fold less tightly than did 1,25-dihydroxy vitamin D<sub>3</sub>. This suggests that 1,24-dihydroxy vitamin D<sub>4</sub> is less toxic than is 1,25-dihydroxy vitamin D<sub>3</sub>. The combination of high biological activity and low toxicity indicates that 1,24-dihydroxy vitamin D<sub>4</sub> would be a useful therapeutic drug and is not predicted or suggested at all in the prior literature. A description of these experiments and the data are provided in Exhibit D hereto, incorporated herein by reference.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date:

August 24, 1994

Joyce C. Knutson  
Joyce C. Knutson

APPENDIX IV

DECLARATION OF ROBERT MORIARTY